SEMMELWEIS EGYETEM

DOKTORI ISKOLA

Ph.D. értekezések

3166.

MÁTÉ-SCHWARCZ DÓRA ANETT

Neuromorfológia és sejtbiológia

című program

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MOLECULAR ANATOMY OF MICROGLIA-NEURON INTERACTIONS

PhD thesis

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Budapest
2025

’’Let us also note that everyone, if they so desire, can become the sculptor of their own brain, and that even the humblest of abilities can yield abundant fruit, like barren land when well cultivated.’’

S. Ramón Y Cayal (1)

’’The most marvelous product of the universe, the human brain, is thus a carrier of an informational world system: our consciousness and everything within it, what we know about ourselves, our language, art, theories, and science—all are carried by functioning brain structures as an informational system.’’

János Szentágothai

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List of Abbreviations

AD	Alzheimer's disease
ADHD	Attention deficit hyperactivity disorder
AIS	Axon initial segment
ALS	Amyotrophic lateral sclerosis
ALSP	Adult-onset leukoencephalopathy
AQP4	Aquaporin-4
ASD	Autism spectrum disorder
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
CA1	Hippocampus cornu Ammonis 1 region
CLEM	Correlated light and electron microscopy
CLSM	Confocal laser scanning microscopy
CNP1	Cyclic-nucleotide 3'-phosphodiesterase
CNS	Central nervous system
CX3CL1	C-X3-C motif chemokine ligand 1 (fractalkine)
CX3CR1	Chemokine (C-X3-C motive) receptor 1 (fractalkine receptor)
CSF-1	Colony stimulating factor 1
DAB	3,3'-Diaminobenzidine
DCX	Doublecortin
DG	Dentate gyrus
E9	Embryonic day 9
EM	Electron microscopy
ER	Endoplasmic reticulum
GABA	Gamma-aminobutyric acid
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
IBA1	Ionized calcium binding adapter molecule 1
IFN	Interferon

IL	Interleukin
Kv	Voltage-gated potassium channel
LAMP1	Lysosomal Associated Membrane Protein 1
MAMs	Mitochondria-associated membranes
MDVs	Mitochondria-derived vesicles
mtDNA	Mitochondrial DNA
NDDs	Neurodevelopmental disorders
NVU	Neurovascular unit
OPCs	Oligodendrocyte precursor cells
P1-90	Postnatal day 1-90
P2Y12R	Purinergic receptor P2Y12
PB	Phosphate buffer
PBS	Phosphate buffered saline solution
PLOSL	Polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy
PNS	Peripheral nervous system
PSB	PSB0739, selective P2Y12 receptor antagonist
Pu.1	Purine-rich box1 (PU. 1) transcription factor
ROS	Reactive oxygen species
SVZ	Subventricular zone
TBI	Traumatic brain injury
TBS	Tris-buffered saline solution
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TOM20	Translocase of outer mitochondrial membrane 20
vGAT	Vesicular GABA Transporter
vGluT1	Vesicular glutamate transporter 1
vNUT	Vesicular nucleotide transporter
VZ	Ventricular zone

1. Introduction

1.1 The role of microglia in the adult nervous system under physiological conditions

The human brain, described by Szentágothai János as the carrier of an informational world system, is the pinnacle of evolution, with 86 billion neurons and a vast network of connections enabling self-awareness and scientific inquiry (2-4). From ancient Hellenistic studies to modern neuroscience, pioneers like Ramón y Cajal and Mihály Lenhossék have shaped our understanding of neuroanatomy, laying the foundation for current research (5,6). Despite groundbreaking discoveries, neurological diseases remain a major global health challenge, with traditional neuron-focused treatments often failing, highlighting the need for broader, translational research approaches (7-10). Recent studies emphasize the role of microglia, the brain's immune cells, in neurodegenerative disorders, acting as "gardeners" by regulating inflammation and maintaining brain health (11-15). Advances in research tools now reveal a more complex role for microglia, shifting the focus toward understanding their physiological functions as potential therapeutic targets for brain diseases (16).

A major breakthrough in determining the true physiological role of microglial cells came with observations performed using two-photon microscopy in living animals, which revealed that the thin processes of microglia — previously described as a "resting" state — continuously and dynamically scan their environment (17,18). While these processes move some µm per minute, the cell bodies under physiological conditions migrate only about 1 µm per day (17,19–21). Consequently, the discovery of dynamic mobility of microglial cells has highlighted that the functions of microglia go far beyond defence against pathogens (22,23).

A widely studied function of microglial cells is to regulate the efficiency of synapses and neuronal circuits (24–28). This includes the adaptation of synapses to conditions, their formation and elimination, as well as the dynamic changes in their size and receptor content—known as synaptic plasticity—which is essential for higher-level cognitive processes (29–31). Microglia play a significant role in reactive synaptogenesis, maturation-, activity- and plasticity of synapses in adulthood (32,33) and in the elimination of unused or defective synapses (34–36). However recent studies conducted

with CSF1RΔFIRE mice - which lack microglia from birth - suggest that microglial role in synaptic pruning may be less critical than previously thought, as no significant changes were observed in hippocampal synapse numbers or spine density (38-40), but suggested that astrocytes may take over the role of synaptic elimination (40–48). Microglia are not only capable of regulating synaptic structures, but also play a key role in the regulation of the myelin sheath, as they induce myelination and phagocytose these structures in a process influenced by neuronal activity (26,49–53). Additionally, they contribute to the proper functioning of the vascular system by regulating cerebral blood flow, vascular remodeling (26,54–56), not to mention their broad roles in different neurological pathologies. Due to their macrophage nature, microglia are responsible for immune surveillance within the brain parenchyma, acting as the primary defense of the central nervous system, which is separated from the periphery by the blood-brain barrier (BBB) (24,57–61). Microglia, in cooperation with astrocytes, have the ability to clear apoptotic cells through two-photon-mediated, photochemically induced apoptosis (62). Last but not least, together with all these functions, microglia contribute to the homeostasis of the brain by regulating the neuronal survival, removing cellular debris and maintaining the balance of the brain's extracellular environment (24,59). Their role in monitoring and responding to local changes provides a stable environment for optimal neural function (63). The diverse range of microglial functions requires, on the one hand, a global network established through the interaction between the periphery and the central nervous system, and on the other hand, a local network sustained by the continuous and dynamic communication between microglial cells and surrounding cells of the brain.

1.1.1 Contactomics of microglia

The densely packed, peripherally isolated brain parenchyma of the central nervous system (CNS) has developed efficient communication strategies to maintain delicate structures and neuronal networks, adapting to the brain's exceptionally high energy demands (64,65). This is supported by microglial cells, which, with their diverse repertoire of receptor and soluble factor expression (66), as well as their mobile processes and sensitive filopodia (17,19,20), continuously perform membrane ruffling and reorganization to ensure the dynamic functionality required for brain operation (67,68). To gain a deeper understanding of microglial function, it is essential to comprehensively map their intricate network of connections, referred to as microglial contactomics (69).

Microglia engage in bidirectional communication on a global scale, connecting CNS with peripheral systems through neuronal pathways or humoral routes (70–72), regulating the blood-brain barrier permeability, essential for maintaining brain integrity, particularly in recruiting peripheral immune cells to the brain following injury (71,73–75) while the microbiome also influences CNS function, as gut flora changes can trigger inflammation, disrupt cerebrovascular health, and alter microglial behaviour and neuronal activity (70–91). This growing research highlights the intricate interplay between biological systems and underscores microglial key role in maintaining brain homeostasis (71).

For the CNS to function optimally, not only global connections but also localized, communication forms of microglia are indispensable. Acting as the "conductors" of the brain's healthy functioning, microglia maintain connections with every cell type in the brain.

1.1.1.1 Connections betweeen microglia and non-neuronal cells

Non-neuronal cellular elements of the CNS include endothelial cells, pericytes, and glial cells, which are further categorized into microglia, oligodendrocytes, and astrocytes. These cells collectively make up half of the brain's cell population, serving as the primary supporters of neuronal cells. There is no consistent information in the literature regarding the precise proportion of glial cells relative to one another due to the varying sensitivity of different methodological approaches. In the human brain, there are approximately equal numbers of neurons (~86 billion) and glial cells (~85-87 billion). Glial cells further divided into astrocytes (~26-30 billion), oligodendrocytes (~39-43 billion), and microglial cells (~12-17 billion), reflecting a fundamentally balanced cellular composition essential for brain function (92–95). However, it is undeniable that the cooperation of these supporting units is essential for maintaining healthy brain function. Consequently, it is evident that the complex interaction between microglia and non-neuronal cells requires well-regulated, bidirectional communication, with direct membrane connections being the most optimal form of such interactions (69). While there is evidence of this in the literature, the temporal dynamics and communication pathways involved have yet to be fully elucidated.

1.1.1.1.1 Oligodendrocytes

A key feature of the nervous system is its rapid transmission of impulses, made possible by the myelin sheath, which is formed by oligodendroglial cells (96). For these

cells to perform their role effectively, research has shown that regulation by microglial cells is necessary, which can occur through direct membrane connections (69). It has been demonstrated that microglia make contact with the myelin sheath at axonal nodes of Ranvier (97), as well as with the outer layers of the myelin sheath (98,99), and electron microscopy has confirmed direct interactions between microglia and oligodendrocyte cell bodies (100,101). The role of microglial cells in myelin modification is so significant that it remains critical from development through ageing. During development, microglia regulate the proliferation of oligodendrocyte precursor cells (OPCs) via direct membrane contacts, thereby contributing to myelination (102), and participate in myelin remodeling through myelin phagocytosis (50,103). With ageing, myelin degradation has been shown to exhaust the clearance capacity of microglia (104,105). Direct microglia-oligodendrocyte cell body contact is also significant in enhancing remyelination (106). However, there is limited information in the literature regarding the ultrastructure of these contacts and the related functions and underlying signaling pathways (69).

1.1.1.1.2 Astrocytes

Astrocytes have complex roles in the CNS. As a component of the blood-brain barrier, they play a crucial part in regulating the vascular system, maintaining brain homeostasis, clearing harmful substances from the brain parenchyma, and ensuring the proper functioning of neurons (96). Numerous examples in the literature illustrate the collaboration between microglial cells and astrocytes, including the quad-partite synapse (107), the removal of neuronal debris (62), their role in epileptogenesis (108), and the development of neuropathic pain (109). They are also involved in each other's regulatory functions, such as microglial regulation of astrocyte differentiation (110) and astroglial clearance of microglial debris (111–113). It has also been shown that microglial cell bodies and processes make direct contact with all parts of astrocytes, including their cell bodies, processes, and endfeet (17,55,69,114–116). Although the underlying signaling pathways remain largely unexplored, the significance of the complement system has been demonstrated in microglial debris phagocytosis by astrocytes and in the development of neuropathic pain (113,117).

1.1.1.1.3 Neurovascular unit

To protect the brain, cerebral circulation must function in a highly specific and finely tuned manner, isolated from peripheral influences. A key feature is the blood-brain

barrier (BBB), which shields neurons from harmful substances and blood fluctuations of ions or other blood components, ensuring stability. Structurally, the brain possesses a unique neurovascular unit (NVU) comprising endothelial cells, pericytes, astrocytes, neurons, and microglial cells. This specialized collaboration allows for dynamic responses to neural activity, ensuring adequate blood supply to brain regions (118).

Microglia-vessel interactions are vital during development, especially in the first postnatal week (119,120), in the regulation of vessel sprouting, anastomosis, and vascular maturation (121,122). Both the literature and our own observations have shown that microglial cells often position themselves at vascular Y-branch points (15,55,115,123). Furthermore, microglial processes continuously survey the vascular wall and establish contact with every member of the neurovascular unit (69). Through these interactions, microglia can seal leaking BBB (124) and modulate blood flow, cerebral hypoperfusion, and neurovascular coupling (55,123,125,129). Microglia regulate angiogenesis and vessel diameters across development and adulthood, often in collaboration with pericytes and smooth muscle cells to control blood flow (55,56,123). Microglia directly interact with over 80% of cortical pericytes, working together to preserve BBB integrity and regulate leukocyte infiltration (115,120,124,126,127,130). In Alzheimer's disease, the disruption of these interactions drives vascular dysfunction, highlighting their critical role in cerebrovascular health (128).

Microglia act as coordinators of non-neuronal cells, ensuring proper CNS function. They form physical connections with nearly all brain cell types, but the functional roles, ultrastructure, and signaling mechanisms of these interactions remain unclear.

1.1.1.2 Microglia-neuron interactions

The interaction between microglia and neurons is a fundamental pillar of healthy brain function, from early development to aging. Microglia actively regulate, support, protect, and monitor neuronal function through both direct and indirect mechanisms. Microglia indirectly regulate neuronal activity by releasing cytokines (TNF-α, IL-1β), neurotrophic factors (BDNF), and other mediators through paracrine or endocrine signaling, often via astrocytes, while neurons reciprocate through ATP, adenosine, purinergic metabolites, and glutamate to modulate microglial responses (18,71,82–84,131–134). While communication mediated by soluble factors allows for the regulation

of distant and extensive cell populations, its effects are less specific and, therefore, less efficient compared to direct connections.

1.1.1.2.1 Direct connections between microglia and neurons

In living organisms, the most precise form of communication between cells occurs through direct membrane connections. The exchange of information is highly regulated in space and time, which is essential for the harmonious cooperation of large numbers of cells. These interactions, through the activation of intracellular signaling pathways, can lead to differentiation, morphological changes, cell migration, or the initiation of apoptosis. The regulation of vital neuronal processes influenced by microglia likely requires direct membrane-to-membrane connections, enabling cells to affect each other using neurotransmitters, ions, membrane-bound receptors, or other integral membrane proteins (23,66). Cells generally exhibit a complex three-dimensional structure with specialized domains or compartments that perform distinct functions. These compartments demonstrate significant functional autonomy in areas such as metabolism, protein synthesis, and signal integration (135). Their stability ranges widely, from static to highly dynamic. The characteristics of neuronal compartments highlight the importance of compartment-specific functional segregation during microglial interactions, suggesting that microglial connections with neurons may vary significantly depending on the subcellular domain involved.

Accordingly, from the microglial perspective, microglial interactions can be classified based on compartment specificity: (I) satellite contacts, where the microglial soma interacts with a cell body of another cell, and (II) process contacts, where microglial processes establish direct membrane connections with an other cell. These interaction types differ significantly, as the movement of the microglial cell body in the brain is considerably slower (1 μm/day) compared to its processes (1-3 μm/min). Satellite-type interactions represent a more static and primitive form of cell communication, similar to the primitive satellite glial cells in the peripheral nervous system, where direct contact with neurons is associated with slower and less precise information transfer (17,19,20,69,136,137).

On the neuronal side, the operation of complex neural networks requires compartment-specific functional segregation in neurons. The key components of this include dendrites, responsible for receiving incoming signals; the somatic compartment,

which processes and modulates these signals; and the axon and its initial segment, which play a crucial role in generating and transmitting action potentials. While the neuronal cell body is relatively stable and stationary, synapses are highly dynamic, forming and disappearing in an activity-dependent manner, demonstrating significant variability. This morpho-functional heterogeneity between compartments necessitates that microglial processes interact differently with each neuronal domain (69,71,96).

1.1.1.2.1.1 Synaptic connections

Microglial interactions with neuronal synapses have gained interest, with microscopy confirming these connections (27,28,33,69,71,136,138). However, only a fraction involve direct membrane contacts, mainly at presynaptic sites, and ~10% of synapses receive microglial input at a time, with contacts lasting 5-10 minutes (28,136,139,140). Microglia regulate synaptic pruning, remodeling, and activity, influencing dendritic spine formation and excitatory-inhibitory balance during development and adulthood (30,37,84,138,141–146). Dysfunctional microglial-synapse interactions contribute to Alzheimer's, multiple sclerosis, ALS, stroke-related synapse loss, and autism-like behaviors (147–151). Several molecular pathways (e.g., phosphatidylserine, C1q, fractalkine, MHC I) regulate synapse elimination and plasticity, though their high-resolution anatomical evidence and signaling mechanisms remain unclear (27,30,33,71,143,152).

1.1.1.2.1.2 Axonal connections

Microglial connections to the axon initial segments (AIS) required for the generation of action potentials are formed in association with the presence of Ankyrin-G scaffolding protein (69,153). During development, netrin signaling guides microglial recruitment to axons, supporting neuronal growth and survival (154). Microglial interactions are also involved in regulating cortical wiring (155) and axonal pruning (156). These direct contacts can modulate synaptic inputs at the AIS by selectively removing synapses from injured neurons (synaptic stripping) (157,158) or influence axo-axonic synaptogenesis (159). Additionally, microglial processes form direct connections at the nodes of Ranvier, facilitating remyelination after injury (97). In cases of traumatic brain injury or axonal hyperactivity, microglial processes cluster around AISs to prevent excessive depolarization (160,161). However, we currently know little about the

signaling background and the compartment-specific functional ultrastructure of these interactions (69,71).

Microglial cells establish short-lived, compartment-specific direct contacts with synapses, dendrites, and axons, regulating there functions. These interactions are essential for the development of neuronal networks, synaptic plasticity, and the repair of damage during injuries and pathological conditions.

1.1.1.2.1.3 Somatic purinergic junctions

The neuronal functions associated with the somatic region are most effectively regulated through the connection with the cell body. Previously, interactions between neuronal cell bodies and microglial processes have been described in relation remove or displace synaptic inputs from the perisomatic region of injured neurons (perisomatic stripping), the phagocytosis of newborn neurons, and neuronal activity (125,162–165). Recently, using various microscopic modalities, we identified a highly specific site of communication between dynamically moving microglial processes and specialized areas of neuronal cell bodies in adult mouse and human brain tissue, which we termed the somatic purinergic junction (136) (Figure 1). Our *in vivo* measurements confirmed that these connections persist significantly longer (25 minutes) than microglial contacts involving neuronal neurites (5-10 minutes) and are present in approximately 90% of cortical neurons at any given time, regardless of neuronal phenotype. These morphofunctional units are uniquely structured at the ultrastructural and molecular levels, optimized to enable dynamic and highly efficient bidirectional communication (136).

Neuronal mitochondria accumulate and anchor at these somatic junctions, alongside mitochondria-associated membranes, endoplasmic reticulum-plasma membrane contacts, and vesicles derived from purinergic and mitochondrial sources (Figure 1). In addition to anatomical observations, functional studies have confirmed that microglia can respond to mitochondrial metabolic activity through somatic junctions (136). The significance lies in the fact that mitochondria, beyond their role in energy production, are involved in various signaling and homeostatic processes such as intracellular calcium flux, proliferation, morphology, antigen presentation, and regulation of cell viability (166–170). Mitochondrial dysfunction, an early indicator of injury, involves membrane permeability changes, ion imbalance, pro-apoptotic signaling, oxidative stress, energy deficits, membrane fragmentation, and mitophagy (136,176).

Microglia detect these changes via somatic junctions (136) and respond to mitochondrial metabolites like reactive oxygen species, ATP, and pro-apoptotic agents, underscoring mitochondrial role in microglial communication (136,171–175). Thus, somatic mitochondria and mitochondria-associated membranes (MAMs) are well-positioned to inform microglia about cellular state (136).

In the somatic junction, we also identified the presence of the lysosomal marker LAMP1 and the vesicular nucleotide transporter vNUT, alongside the accumulation of Kv2.1 and Kv2.2 clusters at neuronal contact sites, indicating their role in the exocytosis process (136) (Figure 1). Kv2.1 proteins regulate K+ currents but, when forming clusters, anchor the endoplasmic reticulum to the membrane, bind SNARE proteins support somatic exocytosis (177–185), thereby facilitating bidirectional communication between the two cells.

The NDTPase enzymes accumulating on the microglia side - which convert ATP - and the purinergic P2Y12 receptors (P2Y12R) are responsible for sensing ATP released from neurons (136) (Figure 1). This receptor, exclusive to microglia in the CNS (186,187), is essential for P2Y12R-dependent responses to increased neuronal activity. Acute inhibition of P2Y12R reduces somatic junction duration and diminishes its neuroprotective effects following ischemic stroke (136). Notably, microglial processes show enhanced coverage of neuronal cell bodies in response to activity, a phenomenon absent when P2Y12R function is impaired. Increased microglial coverage has been observed during experimental strokes and in pathological conditions such as brain slice preparation or COVID-19, indicating its relevance across diverse scenarios (188,189). From a clinical perspective, P2Y12R is particularly significant because inhibitors of this receptor are widely used as anticoagulants following vascular events (190,191). However, these inhibitors may negatively impact microglial function, particularly in cases where the blood-brain barrier is compromised, potentially worsening stroke outcomes.

Microglial processes at somatic junctions may assess neuronal energy states, or metabolic byproducts, enabling an evaluation of cell health and damage severity. The prolonged duration of these connections—sometimes lasting over two hours—suggests a capacity for complex intercellular communication and significant intervention in neuronal function. Anchored by specific proteins, these stable morphofunctional units allow microglial processes to periodically monitor neuronal health, support function, and deliver P2Y12R-dependent neuroprotection after injury (73,136). Observations in adult mouse and human brain tissue raise questions about their presence across the lifespan, from development to aging.

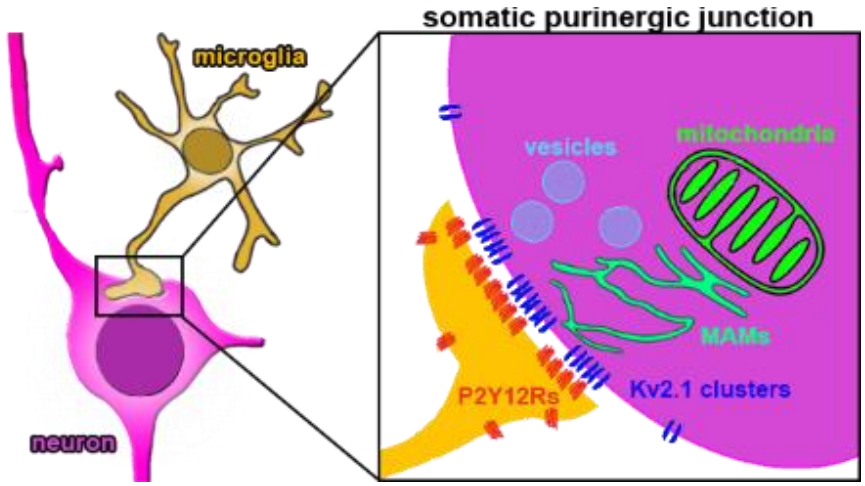

Figure 1. Schematic illustration of the somatic purinergic junction between microglial processes and the cell body of mature neurons identified in the adult mouse and human brain. The insert on the right highlights the key components of the somatic connections. On the microglial side, an accumulation of microglia-specific purinergic P2Y12R receptors can be observed. The neuronal side is characterized by Kv2.1 protein clusters involved in the formation of the exocytic surface, mitochondrial accumulation, vesicular nucleotide transporter (vNUT)-positive, and lysosomal-associated membrane protein 1 (LAMP1)-positive vesicles. Additionally, mitochondria-associated membranes (MAMs) also play a role in the functioning of the junctions.

1.2 Role of microglial cells in development

During development, various cell types proliferate and differentiate, including neurons (neurogenesis), glial cells (gliogenesis), and the cells that form the vascular network (angiogenesis). These cells follow predetermined pathways to their target locations, where they create spatial structures and establish synaptic and other intercellular connections. The precise regulation of these processes is ensured by the

coordinated interaction of genetic programming and environmental factors, which is indispensable for the structural and functional development of the brain (192–196). The pallium of the telencephalic vesicle on embryonic day 9 (in mice) consists of a thin ventricular zone made up of progenitor cells, which are precursors to neurons and glial cells. Initially, neuroepithelial cells divide symmetrically and later begin to differentiate, creating the subventricular zone, with neurons migrating along the radial glial fibers to occupy their positions in the cortical plate (197,198). After neurogenesis concludes, new neurons generally do not form in the mammalian brain, with the exception of the olfactory bulb and the dentate gyrus of the hippocampus in mice, where progenitor cells continue to generate neurons (in the human brain only in the dentate gyrus of the hippocampus) (199). Once individual neurons reach their destinations, their complex spatial structures are finalized, alongside the processes of synaptogenesis and angiogenesis (56). Beyond their role in the adult nervous system, microglia play a crucial role in the development of the central nervous system (87,87,200,201). Microglia are the earliest glial cells to appear in the brain, developing alongside neurons during the crucial stages of early embryonic brain development (202).

1.2.1 Origin and early appearence of microglial cells

The cells of the nervous system, which include neurons, neuroendocrine cells, and glial cells, are derived from the ectoderm, the outer germ layer. In contrast, Pío del Río-Hortega - known as the father of microglia - proposed that microglial cells, originate from the mesoderm. Yolk sac-derived microglial progenitors appear in the CNS as early as the 5th week of gestation in humans and embryonic day 9 (E9) in mice, preceding the colonization of nearly all other cells (56,203–205) (Figure 2). Two potential routes for embryonic microglia to access the brain have been proposed, although they remain unconfirmed. Microglia may either penetrate from the meninges by crossing the pial surface or enter through the ventricles, where they are observed as either free-floating cells or attached to the ventricular wall before migrating into the brain parenchyma. The CX3CL1/CX3CR1 signaling pathway is critical in regulating microglial entry, distribution, and proliferation within the developing brain (56). Early in development, amoeboid microglial cells move tangentially and then radially as they leave blood vessels. Microglial population in the CNS occurs in two main waves. In the first phase, between embryonic day 8.5 and 14.4 in mice, microglial progenitor cells begin to migrate to the

brain, leading to a gradual increase in the population, probably via extravascular pathways due to the lack of vascular network. The second wave, which occurs between embryonic days 14 and 16, involves further cell proliferation and reaches the final density typical of adult brains before the end of embryonic development (56,205–207) (Figure 2). Microglia exhibit significant functional heterogeneity in different brain regions. Their proportion relative to neurons and their transcriptomic profiles vary by location, influenced mainly by the specific microenvironment of each cell (208–211). During embryonic development, microglia are distributed unevenly and form at least four specific hotspots. One population is situated near the radial glial cells in the ventricular zone (VZ) and subventricular zone (SVZ), where microglia assist in regulating the size of the precursor cell pool. Another hotspot forms around new blood vessels, where microglia support angiogenesis. Phagocytic microglia also cluster near dying cells in the choroid plexus and developing hippocampus, where they perform their phagocytic roles. Additionally, microglia are found near developing axons, potentially influencing axonal growth processes (56). Unlike other tissue macrophages in the body, microglia exist within the unique environment of the blood-brain barrier and maintain their population autonomously, without input from circulating immune cells. Their resident population is maintained by a balance between cell division and programmed cell death in which CSF-1 receptors (CSF1R) are essential.(212–217). Their early establishment in the brain parenchyma highlights their crucial functional role in brain development.

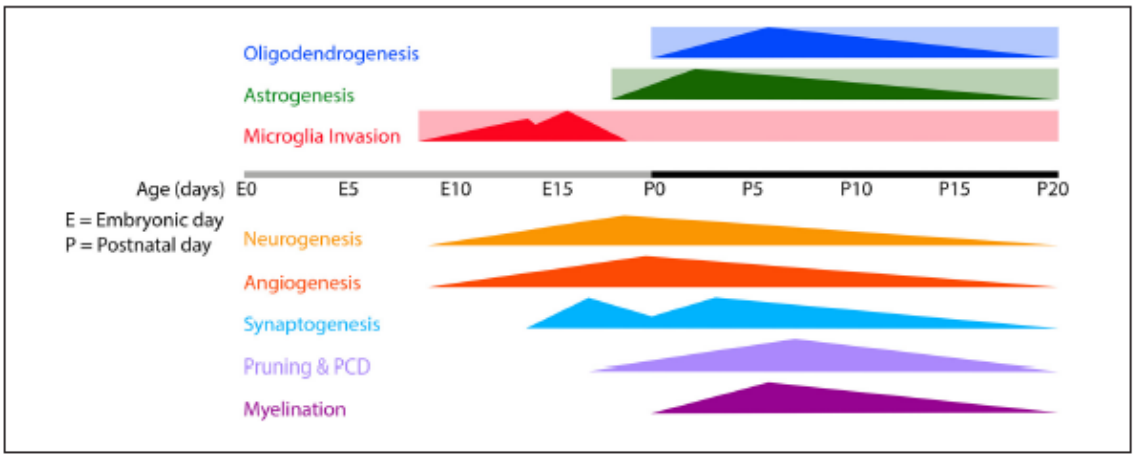

Figure 2. Timeline of the developmental processes in the mouse brain. Dual-wave brain colonization by microglia (red) precedes major developmental stages, supporting its prominent role in the regulation of developmental processes. PCD stands for programmed cell death (Adapted from 56).

1.2.2 Function of microglial cells in development

Reflecting the complexity of brain development, numerous studies show that microglia, due to their early presence in the nervous system, regulate progenitor cell division, migration, and function, contributing to neuro-, glio-, and angiogenesis (122,218–226). The final neuronal count in a healthy brain depends on the balance between neurogenesis and programmed cell death. Microglia support precursor cell proliferation, migration, differentiation and driving programmed neuronal death (227, 230-235), while also promoting neuronal survival (228). This role is especially crucial in early postnatal development, as microglia are essential for the survival of cortical layer 5 neurons (229). After development in adulthood microglia support adult neurogenesis by promoting, regulating, and maintaining neuronal growth, particularly in the hippocampus (236-245). They aid in the survival, integration, and removal of excess neurons postnatally (164).

Of all the functions of microglia in development, the largest body of scientific literature is undoubtedly dealing with their effects on the formation, plasticity and regulation of synaptic connections (236). During development, significantly more synaptic connections are made between developing neurons than the number of synapses observed in the adult brain, of which defective or unused synapses are eliminated (237). Microglia also play a central role in regulating these processes: in various neural processes, including the generation and refinement of new synapses (143,156,238,239), the activity-dependent formation and removal of excess synapses (30,84), synapse and spine remodeling (139), as well as axonal guidance (155,202,240). However, studies using FIRE mice - which lack microglia from birth - question their role in these processes (38–40).

These developmental processes require finely tuned spatial and temporal interactions among microglia, neuronal progenitor cells, and immature neurons, yet the principal sites of cell-to-cell communication and the mechanisms involved remain poorly understood. Communication between immature neurons and microglia can occur through multiple pathways, including indirect intercellular interactions and direct membrane connections as mentioned earlier (71). For instance, microglia have been shown to modulate developmental and adult neurogenesis through the extracellular release of cytokines (IL-1β, IL-6, TNF-α, IFN- γ, TGF-β) and growth factors (186,225,245,246). *In*

vitro experiments have shown that the supernatant collected from microglial cell cultures stimulates the proliferation and neuronal differentiation of stem cells (226). The effect of microglia on intercellular vascular development is supported by *in vitro* experiments where the addition of microglial cells or microglia-derived mediators to endothelial cell cultures enhanced the formation of new blood vessels (122).

In addition to indirect communication, direct interactions with neuronal synapses are vital for the proper development of brain circuits. For example, microglia can interact with dendrites of developing neurons to induce spine outgrowth and promote synaptogenesis (142). The formation of initial axon segments and the suppression of axon outgrowth also occur through direct microglial interaction with growth cones (153,247). Microglial regulation of immature neurons lacking complex synaptic inputs is essential for the formation of functional neuronal networks. However, these interactions do not fully explain how microglia can exert such significant control over the cell fate of developing neurons without a comprehensive assessment of the cell's state through the main control centre, the cell body. The current literature lacks data suggesting the existence of a specific, ultrastructural, bidirectional somatic connection between developing neurons and microglial cells.

1.2.3 Function of microglial cells in developmental disorders

Microglial cells play a crucial role in regulating the formation and functional maturation of developing neural networks, making them significant in the context of neurodevelopmental disorders. However, it often remains unclear whether these disorders are a cause or a result of pathological changes (222,248–251). Neurodevelopmental disorders (NDDs) represent a complex array of conditions that begin in childhood, stemming from some form of disruption in brain development. Given the early onset of neurodevelopmental dysfunctions associated with NDDs and the potential for long-term or even lifelong care requirements, these disorders place a significant social, economic, and medical burden on society. NDDs broadly include a variety of conditions such as autism spectrum disorder (ASD), schizophrenia, attention deficit hyperactivity disorder (ADHD), intellectual disability disorder, Rett syndrome, Down syndrome, cerebral palsy, fetal alcohol spectrum disorders, childhood epilepsy disorders, and various rare genetic disorders impacting development, such as SLC6A1 disease (252–254). Key features of NDDs, such as ASD, often involve impaired functional connectivity between brain

regions, increased cell proliferation, accelerated neuronal differentiation, disrupted synaptic development, and diminished spontaneous and synchronous neuronal activity. These issues can arise due to changes in synapse formation, function, or abnormalities in synaptic transmission (56,255–262). Since microglia regulate many of these processes, it is unsurprising that they are implicated in the pathogenesis of neurodevelopmental disorders. Increasing evidence suggests that mutations associated with NDDs may significantly contribute to ASD development, particularly through their impact on microglial function (258).

Microglia regulate synaptic pruning via the complement cascade (C1q, C3), with ASD-associated PTEN mutations increasing C1q expression and enhancing microglial phagocytosis, potentially contributing to ASD pathology (210,258,263). CX3CR1-deficient microglia fail to support cortical neuron survival, leading to impaired synaptic pruning, reduced connectivity, and ASD-like behaviors, linking CX3CR1 mutations to ASD risk (143,229,258,264). Mitochondrial dysfunction, common in ASD, heightens oxidative stress and immune responses, contributing to neuronal damage and microglial abnormalities. ASD-linked genes like FOXP1 impact mitochondria, affecting cognition and motor function, while increased mitochondrial DNA levels exacerbate neuroinflammation by microglia (258,265-266). Microglial dysfunction is also implicated in Nasu-Hakola disease (PLOSL), caused by DAP12 and TREM2 mutations, leading to axonal demyelination, astrogliosis, dementia, and psychosis. DAP12-deficient mice show long-term synaptic defects, linking prenatal microglial dysfunction to disease progression (56). Microglia loss due to PU.1 transcription factor deficiency disrupts corpus callosum development and impairs brain vasculature (54,201,267,268). Similarly, CSF1R mutations cause severe brain malformations in zebrafish, rodents, and humans, including corpus callosum agenesis and leukoencephalopathy, leading to early mortality or progressive neurodegeneration (42,269–275).

In conclusion, since microglia play a key role in many developmental brain processes, it is reasonable to assume that microglia deficiencies can lead to developmental abnormalities. Thus, microglial dysfunction may be part of the cause of neurodevelopmental disorders and not only a consequence (56,276). Therefore, the identification of microglia-specific therapeutic targets may open new perspectives for the treatment of neurodegenerative diseases.

1.3 Microglial sensitivities to ageing

The role of microglia in neurodegenerative diseases, including age-related conditions, has been a long-standing focus of research. Traditionally, the significant functional changes in microglia observed in such diseases were termed "microglial activation" and associated with "neuroinflammation." However, this terminology is now considered outdated, as in a healthy brain microglia are not merely ''resting'' cells waiting to be ''activated'' by the damage. Instead, they are dynamic and highly active cells that constantly monitor their environment (22,277). Advances in *in vivo* two-photon microscopy have substantiated this view by demonstrating the continuous motility and scanning behaviors of microglia (17,18,278).

With age, the immune system shifts toward a pro-inflammatory state known as "inflamm-aging" (279,280), reducing microglial efficiency and leading to dysfunctional or hyper-reactive responses (278–280). This decline weakens their protective role, allowing harmful agents to accumulate and exacerbating neurodegeneration, especially in response to infections. Microglia may "age" due to cumulative exposure to systemic infections and environmental factors, leading to exaggerated immune responses. Neurodegenerative diseases represent an extreme state of microglial dysfunction, influenced by genetic predisposition and risk factors like smoking, hypertension, and diabetes (278,281). While ageing and neurodegenerative microglial phenotypes differ, chronic stimulation combined with these risk factors greatly increases neurodegeneration risk (278,280).

Where do we draw the line between physiological ageing and neurodegenerative disease? Ageing is not a disease, but a natural, progressive process marked by anatomical and functional decline, often accompanied by cognitive impairment. While numerous changes associated with ageing have been identified, there is still no consensus on a unified definition of normal ageing (282). In humans, distinguishing normal ageing from abnormal ageing often involves factors such as CNS damage, the presence of amyloid plaques, neurodegeneration, neuropsychiatric conditions, dementia, and declining performance on cognitive tests (283,284). A practical definition of normal ageing might include the absence of overt pathology and dementia, alongside the presence of cognitive decline typical of the ageing population. However, there is no agreement on when

cognitive decline begins, as this varies depending on factors such as age, gender, individual differences, and analytical methods (278,285).

The uncertainties about the relationship between ageing and microglia are greatly aided by the work of Lopéz-Otén and colleagues, who attempted to identify and categorize the "aging features" of microglia: (1) proliferation; (2) morphological remodeling; (3) motility and migration; (4) intercellular communication; (5) phagocytosis; and (6) proteostasis (278,286,287).

One of the key characteristics of microglia is their self-renewal capacity, which is expected to be preserved during ageing. Human microglia are renewed at an annual rate of approximately 28%, with an average cell age of 4.2 years. Over a lifetime, more than 96% of the microglial population is renewed, ensuring continuous replenishment and maintenance of these cells (288). Stable cell density is achieved through finely tuned regulation of proliferative and apoptotic processes. While microglial proliferation increases in response to injury signals, it operates at a lower rate under normal physiological conditions (212,289-292). However, it has been observed that the proliferative capacity of microglial cells declines with ageing (285). If microglia are long-lived cells, it raises the question of how chronic or even occasional inflammatory stimulation might affect them over a lifetime. Similar to other somatic cells, microglia seem to undergo replicative senescence, which is associated with telomere shortening (293), one of the two well-known "hallmarks" of ageing (286). Senescent cells are typically under strict immune surveillance and are removed through phagocytosis. Neighboring microglial cells can engulf and clear apoptotic microglia to prevent the release of pro-inflammatory substances and maintain tissue health (294). Furthermore, microglia can regulate their numbers through autophagy, a process that breaks down and recycles cellular components, supporting cellular health and preventing an overactive immune response (295). This raises the additional question of whether microglia, like neural stem cells, might suffer a loss of regenerative capacity, shifting the balance toward a greater proportion of ageing and dysfunctional microglia, accompanied by fewer protective immune cells. As a result, the ageing brain may become less capable of mounting an effective immune response when faced with injury or neurodegenerative disease (278).

Morphological changes in microglia were among the earliest indicators of aberrant activation states in CNS diseases. These changes are typically marked by an initial retraction of branching processes and mild hypertrophy (296). For instance, in Alzheimer's disease, microglia are well-documented to exhibit a “reactive” morphology characterized by short, thick, and poorly branched processes (297). The effects of ageing on microglial morphology are less understood, but human studies identify dystrophic microglia with fragmented, tortuous processes (285), whereas rodents show reduced arborization, lower complexity, and soma size variability (125,298–300). These differences suggest microglial heterogeneity and emphasize the need for integrated human and rodent studies for future translational applications. The molecular mechanisms behind these morphological changes remain largely unknown, requiring further research to link them to specific pathological conditions (278,280).

As previously mentioned, microglial projections actively and dynamically scan the brain parenchyma. However, microglial motility generally declines with age (298,299), potentially signaling a reduction in immune surveillance. This is corroborated by studies showing diminished responses of aged microglia to stimuli such as exogenous ATP, focal tissue injury, and amyloid pathology. In aged animals, these responses are characterized by reduced elongation, impaired cell migration, and thickened projections of microglia (285). Further evidence for age-related changes in microglial dynamics comes from transcriptomic analyses, which reveal that young microglia are more enriched in motility-related genes (301). The integrity of microglial motility is crucial for their physiological functions, including maintaining homeostasis and responding to injury,but age-related decline impair these functions, weakening both baseline regulation and recovery processes (278).

Microglial signaling molecule expression varies greatly based on environment and cell state. Earlier studies erroneously classified microglia into M1 (pro-inflammatory) and M2 (anti-inflammatory) states, but they are now recognized as a highly heterogeneous population (22,302–304). Increased expression of antigen presentation, lysosomal, and pathogen-recognition proteins is linked to reactive microglia (301). While these responses help defend the brain, losing balance can lead to irreversible dysfunction, especially with aging. Test results vary widely, depending on pathology. For instance, IL-4R levels decrease in aged mice after traumatic brain injury (TBI) but increase under LPS-induced

immune challenge (305,306). Some studies found slower M2 gene induction in aged animals after cytokine exposure or hemorrhage (307), while others found no age-related microglial activation differences without pathology (308,309). Disrupting the balance between stimulation and restraint may trigger excessive immune responses, leading to neuronal damage via pro-inflammatory cytokines and oxidative stress. However, the mechanisms behind these phenotypic changes remain unclear (278, 310).

Among the characteristics of microglial ageing, the effects on phagocytosis remain one of the least clearly defined. Studies in rodent brains indicate that senescent microglia accumulate various non-cellular inclusions, such as vacuoles, large vesicles, lysosomal inclusions, and condensed debris, likely lipofuscin pigment granules (311), which have been associated with significant deficits in phagocytosis (312). Aging differentially impacts microglial phagocytosis of myelin, Aβ, and cellular debris, as well as synaptic pruning potentially contributing to neurodegenerative diseases like Alzheimer's, where declining Aβ clearance due to receptor and enzyme reduction accelerates synaptic loss and cognitive decline (33,99,104, 313–320). Impaired phagocytic efficiency in ageing microglia may result from various factors, including reduced target recognition due to receptor downregulation, impaired chemotaxis leading to delayed migration to injury sites, or saturation with degradative substances. Beyond these initial steps, it also disrupts particle ingestion, phagosome-lysosome fusion, and material clearance. Future research should enhance clearance mechanisms, explore phagocytosis stages, and regulate degradation of proteins within cells (proteostasis) to understand microglial aging and its role in disease progression, potentially guiding new therapies (278).

The microglial ageing "features" - reduced proliferative capacity, dystrophy, loss of mobility, altered signal transduction, impaired phagocytosis and proteostasis - that Lopéz-Otín has identified are well suited to the separation and in-depth investigation of natural ageing and related neurodegenerative diseases. However, it is important to note that all these functions are inherently interrelated and that the profile of one response is likely to influence the outcome of the other response. The majority of publications on the microglial-ageing relationship indicate what microglial ageing looks like, but not why it occurs. Understanding the mechanisms underlying these features will be crucial for designing future interventions to halt or even reverse the progression and mitigate the effects of microglial ageing on the surrounding neural tissue (278, 321-324).

2. Objectives

Microglial cells contribute to diverse processes in the CNS via interactions with multiple cell types. In the adult CNS, we have been able to identify a morphofunctional unit with a specific ultrastructure that is formed between the microglial processes and the cell body of neurons, through which microglia modulate neuronal function in adulthood. However, the role of these interactions during development is not known. Therefore, we were curious whether microglial cells, which are the first to appear in the brain parenchyma, might be able to regulate the development of immature neurons through somatic junctions. In line with this, we also set out to study the role of microglia during the ageing process and changes in somatic connections with neurons. Understanding the physiological role of microglia-neuron interactions may also be important to understand mechanisms of developmental disorders and age-related pathologies.

In our experiments, we therefore aimed to answer the following questions:

in development:

1. Can a direct somatic membrane-to-membrane connection be found between the cell body of developing neurons and microglia, both during development and during adult neurogenesis?
2. Are the specific ultrastructural and cellular components previously identified in somatic connections in mature neurons also found in immature neurons?
3. What might be the role of the somatic connections during development under physiological conditions?

in ageing:

4. Does the distribution of cells change during aging?
5. What are the types and prevalence of connections between microglia and other brain cells in the adult cortex, and how do these change with ageing?
6. Are there any changes in somatic connections over time?
7. Is there any functional change in the two-way microglia-neuron communication during the aging process?

3. Methods

Several subsections of the methods are based on those described in Cserép and Schwarcz et al., 2022.

3.1 Post mortem human samples

For our experiments on human brain tissues, the samples were taken from middle-aged (aged 55 and 60) and the aged individuals (aged 79 and 85) who had died from causes unrelated to brain abnormality (ethical approvals: ETT-TUKEB 62031/2015/EKU, 34/2016 and 31443/2011/EKU [518/PI/11]). Patient data are summarized in Table 1. The use of tissues for research and access to medical data for studies was based on informed consent. Patient identity was kept fully anonymous throughout the study. Tissue samples were handled, stored and used in accordance with the Declaration of Helsinki.

Table 1. Human patient data from the study

Patient ID	Gender	Age (year)	Known neuropathology	Cause of death
SKO7	male	55	no	pulmonary embolism with acute cor pulmonale
SKO13	female	60	no	respiratory arrest
SKO24	male	79	mild small vessel disease (SVD), primary age related tauopathy (PART) in amygdala	left ventricular failure
SKO18	male	85	mild small vessel disease (SVD), moderately severe cerebral amyloid angiopathy (CAA), age-related tau astrogliopathy (ARTAG)	congestive heart failure

The brains of patients who died of non-neurological causes were removed 3-5 hours after death. After cannulation of the vertebral and internal carotid arteries, the brains were perfused with physiological saline containing heparin (approximately 1.5 l solution over 30 min), followed by a fixative solution containing 4% paraformaldehyde (PFA), 0.05% glutaraldehyde and 0.2% picric acid (4-5 l solution over 1.5-2 h). After perfusion, tissue sections containing cortical and hippocampal regions were kept in a glutaraldehyde-free

fixative solution for an additional day, and then 50µm-thick sections were made using a vibratome (VT1200S, Leica Biosystems).

3.2 Ethical statement

All experiments were performed in accordance with the Codex of Ethics of the Research Institute of Experimental Medicine (KOKI) and the Hungarian Act of Animal Care and Experimentation guidelines (40/2013, II.14), which are in concert with the European Communities Council Directive of September 22, 2010 (2010/63/EU). The Animal Care and Experimentation Committee of the Institute of Experimental Medicine and the Animal Health and Food Control Station, Budapest, have also approved the experiments under the number PE/EA/1021–7/2019, PE/EA/673–7/2019.

3.3 Animals

In all experiments, male C57Bl/6J mice were used, from the appropriate age group for the experiment (RRID:IMSR_JAX:000664). For developmental studies, we used embryos at day 15 of embryonic development (E15), young mice at 1 (P1), 8 (P8) and 15 days of age (P15) after birth, and adult animals at 90 days of age (P90). We used 90-day-old and 600-day-old mice for our ageing experiments. Several experiments were carried out on P1 and P8 old CX3CR1$^{+/GFP}$ (IMSR_JAX:005582), P8 old P2Y12$^{-/-}$ (325) and CX3CR1$^{GFP/GFP}$ male mice. For the *in vivo* experiments, male CX3CR1$^{+/tdTomato}$//Thy1/gcamp6$^{+/GFP}$ mice were studied from 50-60 days of age until 650-750 days of age. Cx3CR1 $^{+/GFP}$ mice were used for the *in vivo* mitochondrial measurement. Animals were bred at the SPF unit of the Animal Care Unit of the Institute of Experimental Medicine (IEM, Budapest, Hungary). Mice had free access to food and water and were housed under light-, humidity- and temperature-controlled conditions.

3.4 Perfusion and tissue processing for histology

Mice were anesthetized by intraperitoneal injection of 0.15–0.25 mL of an anesthetic mixture (containing 20 mg/mL ketamine, 4 mg/mL xylazine-hydrochloride). Animals were perfused transcardially with 0.9% NaCl solution for 1 min, followed by 4% freshly depolymerized PFA in 0.1 M phosphate buffer (PB) pH 7.4 for 40 min, and finally with 0.1 M PB for 10 min to wash the fixative out. Blocks containing the primary somatosensory cortex and dorsal hippocampi were dissected and coronal sections were prepared on a vibratome at 50 µm thickness for immunofluorescent experiments and

electron microscopy. For some experiments, 25 µm-thick sections from E15 mouse brains were prepared on a cryostat, and dried onto glass slides.

3.5 Immunofluorescent labeling and CLSM

Before immunofluorescent staining, 50 µm-thick sections were washed in PB and TBS. For Ctip2 and Satb2 stainings, citrate-buffer treatment (10 mM Sodium-citrate, pH 6.0, 90°C, 45 min) was applied. After blocking in 1% HSA and 0.03–0.1% Triton X-100 (except for Ctip2 and Satb2), sections were incubated overnight with primary antibodies at room temperature, followed by secondary antibody incubation at 4°C. If nuclear labeling was required, samples were treated with DAPI and mounted with Aqua-Poly/Mount. TUNEL assay was performed using the Apoptag Red In Situ Apoptosis Detection Kit, and imaging analysis was conducted with Fiji software. The primary and secondary antibodies used are listed in Table 2.

Table 2. List of primary and secondary antibodies used for experiments

ANTIBOIDES	HOST	SOURCE	IDENTIFICATION
PRIMARY ANTIBODIES			
Annexin V	rabbit	Novusbio	Cat# NB100-1930, RRID: AB_10001784
Aquaporin4	guinea-pig	Synaptic Systems	Cat# 429-004, RRID: AB_2802156
CNP1	rabbit	Synaptic Systems	Cat# 355-003, RRID: AB_2620112
Ctip2	rabbit	Abcam	Cat# AB18465, RRID: AB_10973033
DCX	mouse	Santa Cruz	Cat# sc-271390, RRID:AB_10610966
GFP	chicken	Invitrogen	Cat# A10262, RRID:AB_2534023
GFAP	mouse	Sigma	Cat# G3893, RRID:AB_477010
Homer1	rabbit	Synaptic Systems	Cat# 160003, RRID:AB_887730
IBA1	guinea-pig	Synaptic Systems	Cat# 234004, RRID: AB_2493179
IBA1	goat	Novusbio	Cat# NB100-1028, RRID:AB_521594
IBA1	rabbit	Wako Chemicals	Cat# 019-19741, RRID: AB_839504
IBA1	chicken	Synaptic Systems	Cat# 234-009, RRID: 234009
Ki67	rabbit	Abcam	Cat# ab15580, RRID: AB_443209
Kv2.1	mouse	NeuroMab	Cat# 75-014, RRID: AB_10673392
Kv2.1	rabbit	Synaptic Systems	Cat# 231 002 RRID: AB_2131650
LAMP1	rabbit	Abcam	Cat# ab24170, RRID: AB_775978
Lectin	biotinylated	Vectorlabs	Cat# B-1175, RRID: AB_2315475
MAP2	guinea-pig	Synaptic Systems	Cat# 188-004, RRID: AB_2138181

NeuN	mouse	Millipore	Cat# MAB377 RRID: AB_2298772
P2Y12R	rabbit	Anaspec	Cat# AS-55043A, RRID:AB_2298886
Satb2	mouse	Abcam	Cat# AB51502, RRID:AB_882455
SCL17A9	rabbit	Alomone Labs	Cat# ANT-085, RRID: AB_2827340
TOM20	rabbit	Santa Cruz	Cat# sc-11415, RRID:AB_2207533
TOM20	mouse	Abnova	Cat# H00009804-M01 RRID: AB_1507602
vGluT1	guinea-pig	Synaptic Systems	Cat# 135304, RRID: AB_887878
SECONDARY ANTIBODIES			
biotinylated anti-rabbit	donkey	BioRad	Cat# 644008, RRID:AB_619842
1.4 nm Nanogold®-Fab' anti-rabbit IgG	goat	Nanoprobes	Cat# 2004Cat# 2004 RRID: AB_2631182
Alexa 488 anti-goat	donkey	Jackson	Cat# 705-546-147, RRID:AB_2340430
Alexa 488 anti-guinea-pig	donkey	Jackson	Cat# 706-546-148, RRID: AB_2340473
Alexa 488 anti-chicken	donkey	Jackson	Cat# 703-546-155, RRID:AB_2340376
Alexa 488 anti-rabbit	donkey	Jackson	Cat# 711-546-152, RRID:AB_2340619
Alexa 594 anti-guinea-pig	goat	LifeTech	Cat# A11076, RRID:AB_141930
Alexa 594 anti-rabbit	donkey	LifeTech	Cat# A21207, RRID:AB_141637
Alexa 594 anti-chicken	donkey	Jackson	Cat# 703-586-155, RRID: AB_2340378
Alexa 594 anti-mouse	donkey	Invitrogen	Cat# A-21203, RRID:AB_141633
Alexa 594 anti-rat	donkey	Jackson	Cat# 712-586-153, RRID:AB_2340691
Alexa 647 anti-guinea-pig	donkey	Jackson	Cat# 706-606-148, RRID:AB_2340477
Alexa 647 anti-chicken	donkey	Jackson	Cat# 703-606-155, RRID: AB_2340380
Alexa 647 anti-rabbit	donkey	Jackson	Cat# 711-605-152, RRID:AB_2492288
Alexa 647 anti-mouse	donkey	Jackson	Cat# 715-605-150, RRID:AB_2340866

3.6 *In vivo* surgeries

3.6.1 *In utero* electroporation

Female C57Bl/6J (RRID: IMSR_JAX:000664) mice were bred with male homozygous CX3CR1$^{GFP/GFP}$ (RRID:IMSR_JAX:005582; (326) mice. On day 14.5 of embryonic development, pregnant females were anesthetized with isoflurane, and their abdominal cavities were opened to dissect the cornu uteri. Approximately 1 µl of the expression vector Mito-R-Geco1 (1 µg/µl solution) dissolved in endotoxin-free water with added Fast Green dye (1:10000) was prepared. This solution was injected into the embryonic side chambers using a glass capillary. Electroporation was carried out with the In Utero Electroporator SP-3c (Supertech, London, UK) into the somatosensory region of the cortex, using five 50V pulses, each lasting 50 ms with 950 ms intervals. After

electroporation, the cornu uteri were repositioned into the abdominal cavity, and the wound was sutured, closing both the muscle walls and skin. The embryos were then allowed to develop and be delivered naturally.

3.6.2 Cranial window surgery

Mice were anesthetized with fentanyl (100-200 µl) for this procedure. A cranial window, 3 mm in diameter, was created over the primary somatosensory and supplementary motor cortical regions of the left hemisphere, centered 3 mm lateral and 2 mm posterior to Bregma, while keeping the dura mater intact. Following the removal of a section of the cranial bone, a combined 3-mm and 5-mm circular glass cover slip assembly was positioned on the dura surface and secured using Vetbond tissue adhesive (3M, Two Harbors, MN, USA). A custom-made metallic retainer (Femtonics Ltd., Budapest) was then affixed around the glass cover using medical cement.

3.7 Microscopy

3.7.1 Slide scanner microscope imaging

For cell counting, we used a Pannoramic MIDI II/2 slide scanner (3DHISTECH, Budapest, Hungary) equipped with a Zeiss Plan-Apochromat 20× objective lens (NA: 0.8), a Lumencor SPECTRA X light engine with LED illumination and PCO.edge 4.2 back illuminated sCMOS camera. We used: DAPI-1160B, TRITC-Q, FITC-2024B, LF635-C, Cy3.5 filters. Scanning was performed in z-stack mode with step size of 2 µm and pixel size of 6.5 µm x 6.5 µm. The images were analyzed using SlideViewer software version 2.5.

3.7.1.1 Quantitative analysis of cell number distribution

For cell counting, slide scanning microscopy images were taken of whole sections of C57Bl/6J P90 and P600 day-old mice and human samples. ROIs (125 x 125 µm) of the same size were uniformly distributed in the cortical areas throughout the entire thickness of the cortex, and cell bodies with the entire range of cells within the ROI were counted according to the optical disector of the stereological measurement rules.

3.7.2 Confocal Laser Scanning Microscope (CLSM) imaging

Immunofluorescence was analyzed using a Nikon Eclipse Ti-E inverted microscope (Nikon Instruments Europe B.V., Amsterdam, The Netherlands), with a CFI Plan Apochromat VC 60X oil immersion objective (NA: 1.4) and an A1R laser confocal

system. 405, 488, 561 and 647 nm lasers (CVI Melles Griot) were used, and scanning was done in line serial mode. Image stacks were taken with NIS-Elements AR. For each measurement type, image sequences with a resolution of 50 nm/pixel and a step size of 0.5 µm in the z direction were used.

3.7.2.1 Quantitative analysis of CLSM data

Quantitative analysis of each dataset was performed by at least two observers, who were blinded to the origin of the samples, the experiments and did not know of each other's results.

3.7.2.1.1 Colocalisation measurement of microglial markers

For the colocalization measurement of microgial markers during development in the mouse brain, confocal stacks with triple immunofluorescent labeling (IBA1-Gp, IBA1-Gt and P2Y12R) were used, acquired from the VZ/SVZ region of E15, the neocortex of P1-P15 and the dentate gyrus of P90 mice. During the analysis, cells with a DAPI-labeled nucleus in the IBA1-Gp channel were randomly selected. After that the colocalization was measured with the other two microglial labelings (IBA1-Gt, P2Y12R) in each age group.

3.7.2.1.2 TOM20 fluorescent intensity measurement

TOM20 fluorescence intensity was analyzed using a semi-automatic method. Confocal stacks (microglia, DCX, TOM20) were collected, and the largest neuronal cross-section was used to trace the DCX-labeled membrane. This contour was expanded/narrowed by 0.5 µm to define extracellular and intracellular lines for fluorescence analysis. Microglial contact was identified where its intensity exceeded 20% for at least 500 nm, and TOM20 intensity was measured within a 500 nm extended contact area.

3.7.2.1.3 Measurement of the density of DCX-positive cells

DCX+ cell density in the P8 developing cortex was measured using Ctip2 and Satb2 staining to define cortical layers. Cells in layers 6, 4/5, and 2/3 were counted using the optical dissector stereology method. A 50 µm-thick cortical section was imaged with CLSM to create a 3D z-stack. Cells were counted within 50 × 50 µm ROIs using NIS-Elements software, following stereological principles.

3.7.2.1.4 Measurement of the density of Kv2.1-positive cells

For the measurement of the density of Kv2.1-positive neuronal cell bodies in the adult cortex of different genotypes, Kv2.1 immunofluorescent staining, together with DAPI-labeling were used to delineate cortical layers (as described in (327), and the density of neurons was assessed in layers 6, 5, 4, 2/3 and 1. Neuronal cell bodies were counted with the optical dissector method within the 50 µm thick CLSM stacks, using the NIS-Elements software and 50 x 50 µm counting frames.

3.7.2.1.5 Measurement of Annexin-V- and Ki67-positive cells

DCX+ cell distribution, Ki67+ and Annexin-V+ cell density, their colocalization with DCX, and microglial contacts were analyzed in P1 cortex using 20 µm cryostat sections from WT and P2Y12R -/- mice. The cortex width was divided into 10 equal parts for analysis (Figures 11B, C). Objects within these ROIs were counted using the optical dissector method. Measurements were taken from sections with identical rostro-caudal positions.

3.7.2.1.6 Somatic junction prevalence measurement

Somatic junction prevalence was analyzed using confocal stacks with Kv2.1 and IBA1 labeling in P90/P600 mouse neocortex and human Br17 cortex. Only fully captured cell bodies within the Z-stack were counted. Neurons were randomly selected based on neuronal signals, and microglial contact was confirmed when a process touched the soma without a gap for at least 0.5 µm.

3.7.2.1.7 Measurement of microglial contactology

Confocal Z-stacks with 2 different immunofluorescent labelings (microglia, neuron, astroglia, nucleus, and microglia, oligodendroglia, nucleus) were collected. Randomly selected microglia were traced, and their contacts with neurons, astrocytes, oligodendrocytes, and blood vessels were marked. Contacts were categorized as process-process, process-cell body, or cell body-cell body (satellite) interactions. Additionally, contacts to microglial cell bodies were counted (Figure 14).

3.7.2.1.8 Measurement of microglial coverage of nerve cells

Microglial coverage was measured using confocal images with neuronal and microglial signals. Neurons were blindly selected, ensuring full visibility of their cell bodies. Each soma was 3D reconstructed, and its perimeter measured on every plane using NIS-

Elements software. Microglial contact area was also measured per plane, with coverage expressed as a percentage of the total neuronal surface to account for section shrinkage.

3.7.2.1.9 Measurement of TOM20, VNUT, and Kv2.1 marker localisation

TOM20 and VNUT localization were analyzed using confocal z-stacks with triple labeling (microglia, Kv2.1 neurons, and TOM20 or VNUT). Somatic junctions were randomly selected based on neuronal and microglial signals, with contact confirmed if a microglial process touched the soma for at least 0.5 µm. TOM20 and VNUT signals were identified within the neuronal cytoplasm at a 2 µm depth from the membrane. VNUT signal was also associated with Kv2.1 protein clustering along the somatic junction.

3.7.3 Correlated CLSM and immune-electron microscopy

During the immunohistochemical procedure described in Chapter 3.5, chicken anti-DCX, guinea-pig anti-IBA1, together with either rabbit anti-IBA1 or rabbit anti-P2Y12R primary antibodies and fluorophore-conjugated and biotinylated secondary antibodies were used. Sections were mounted in PB, coverslipped, sealed with nail polish. Immunofluorescence for DCX and IBA1-Gp was analyzed using CLSM, with image maps created for electron microscopy correlation. Samples were trimmed, incubated in ABC complex, and the immunoperoxidase reaction developed using DAB. Sections were treated with OsO_4, dehydrated, dehydrated in ascending alcohol series, 1% uranyl acetate in 70% ethanol and in acetonitrile and embedded in Durcupan, and ultrathin 70 nm sections were prepared for electron microscopy. For electron microscopic analysis, correlated tissue samples from the VZ of E15 and from the gyrus dentatus of P90 mice were glued onto Durcupan blocks. Consecutive 70nm-thick sections were cut using an ultramicrotome (Leica EM UC7) and picked up on Formvar-coated single-slot grids. Ultrathin sections were examined in a Hitachi 7100 electron microscope equipped with a Veleta CCD camera (Olympus Soft Imaging Solutions, Germany). During the correlation, a large number of widefield images were taken from the samples and several maps were constructed to ensure that the very same tissue volumes are processed for electron microscopy that have been imaged using CLSM. Correlated images ensured the same tissue volumes were analyzed in CLSM and EM, identifying 10 somatic contacts in an adult and 7 in an E15 animal.

3.7.4 *Ex vivo* 2-photon imaging in acute brain slices

Acute brain slices were prepared from 1- and 8-day-old (P1 and P8) CX3CR1+/GFP mice. Animals were placed on ice for 5–6 min, decapitated and the brains were transferred rapidly to ice-cold artificial cerebrospinal fluid (ACSF; containing in mM: 126 NaCl, 2.5 KCl, 10 glucose, 1.25 NaH_2PO_4, 2 $MgCl_2$, 2 $CaCl_2$ and 26 $NaHCO_3$) saturated with carbogen (95% O_2-5% CO_2). Coronal (300µm-thick) brain slices were cut with a Leica VT1200S Vibratome in ice-cold ACSF. The slices were loaded with 40 mM Cal-590-AM dissolved in ACSF, for 60–90 min, at RT. The Calbryte 590-AM, this robust fluorescence-based assay tool for detecting intracellular calcium mobilization was chosen for its brightness, high signal to noise ratio, improved intracellular retention and loading efficiency as well as homogenous cytoplasmic distribution. Slices were continuously oxygenated during dye incubation. Multiphoton imaging was performed using a Nikon Eclipse FN1 microscope with a 980 nm excitation wavelength to simultaneously measure GFP and Cal-590 signals. The Z-stack (2 µm step size, 10 µm range) images were taken at a 0.25 frame per second rate, for 15 min in ACSF (3 mL/min perfusion rate) followed by a 15 min perfusion with vehicle or 4 mM PSB-0739, a selective P2Y12R antagonist. Calcium data were analyzed using Fiji, with microglial process coverage determined in a 1.2 µm region around each neuron, comparing control vs. PSB-0739 (4 mM) treatment (Figures 9E, G).

3.7.5 *In vivo* 2-photon imaging in live animals

Experiments used a Femto2D-Dual Scanhead microscope (Femtonics Ltd.) with a Chameleon Discovery tunable laser (Coherent, USA) set to 920 nm for GFP, GCaMP6f, and tdTomato excitation. Imaging was performed with resonant and galvo scanning modes using a Nikon 18x water-immersion objective, with data acquired via MES and MESc software (Femtonics Ltd.). To minimize potential interference from volatile anesthetics on microglial process motility, as suggested by recent findings (328), fentanyl anesthesia was selected for these studies. This choice ensured unaffected microglial responses (Fig. S5b; median process motility: isoflurane 0.6 µm/min, interquartile range 0.3–0.83; fentanyl 0.6 µm/min, 0.42–0.78; urethane 0.48 µm/min, 0.36–0.84; n=153 processes from 9 animals). Galvano Z-stacks (7 images, 5 µm steps, 820×820 pixels) were taken every 2–2.5 min across 200–225 µm depth. Two-photon images were analyzed in FIJI, with dual-color tracking performed using FIJI's Manual Tracking

plugin. For microglial process-neuronal mitochondria visualization, Mito-R-Geco1-electroporated CX3CR1+/GFP mice were imaged with a 1000 nm laser.

3.7.5.1 Longitudinal imaging and quantification

We developed a protocol for longitudinal in vivo cell measurement in CX3CR1+/tdTomato//Thy1/gcamp6+/GFP mice, starting at 50–60 days of age, 1–2 weeks post-craniotomy. Imaging areas were mapped using blood vessels and neurons for precise relocation. Resonant recordings (6 × 3 min) were taken in a single plane, with galvo Z-stacks (±50 µm, 3 µm steps). Measurements continued bi-monthly until mice reached 650–750 days. Cells with somatic connections and calcium activity were selected and fluorescence intensity was analyzed in MESc software. Neuronal calcium events that showed at least a 20% increase in fluorescence intensity were tested. Changes were defined as an increase or decrease in microglial process fluorescence intensity observed in the 1.5-min interval before and after the calcium peak. In the analysis, we examined the percentage of the two fluorescence signals that showed a simultaneous change.

3.8 Statistical analysis

All quantitative assessment was performed in a blinded manner wherever it was possible. We applied the nonparametric Wilcoxon signed-rank test to compare two dependent data groups, the Mann-Whitney U-test to compare two independent groups, and the Kruskal-Wallis test to compare multiple independent groups. Statistical analysis was performed with the Statistica 13.4.0.14 package (TIBCO), differences with $p < 0.05$ were considered significant throughout this study. (Significance was labeled on the figures the following way: not significant: n.s., $p < 0.05$: *, $p < 0.01$: **, $p < 0.001$: ***, $p < 0.0001$: ****).

For the preparation of human sample fixation among the listed experimental methods, I received assistance from outside the research group, while for the *in vitro* experiments were performed by members of our research groupe.

Among the results presented in this thesis, I played a major role in the design of experiments, perfusion of animals, sample preparation, in vivo experiments, microscopic imaging (confocal, slide scanning, electron, 2-photon microscopy). Additionally, I contributed to establishing the correlated CLSM and immuno-electron microscopy methods, optimizing the in vivo longitudinal measurement protocol, developing measurement techniques, and analyzing the images and data.

4. Results

4.1 The importance of microglia-neuron somatic junctions in development

4.1.1 Microglia-neuron interactions in development

Microglia, as one of the first cells to colonize the central nervous system, play a pivotal role in ensuring healthy brain development from the earliest stages. They regulate neurons from their genesis (neurogenesis, proliferation) to their maturation (differentiation, migration, apoptosis, and survival). However, the specific mechanisms of cell-cell interactions through which microglia execute these functions remain incompletely understood. The primary aim of our research was to explore this question. To investigate this, we analyzed samples from embryonic E15 mice (ventricular zone [VZ]/subventricular zone [SVZ]) and early postnatal mice (postnatal days 1 [P1], P8, and P15) during developmental neurogenesis, focusing on the neocortex. For adult neurogenesis, we examined the subgranular zone of the dentate gyrus (DG), a neurogenic niche that persists into adulthood (330). Microglia were identified based on the expression of the P2Y12 receptor (P2Y12R) and IBA1, markers known from the literature to be present in yolk sac-derived microglia from the earliest embryonic stages (331–333). To validate the expression levels and localization of these markers, we used multiplex immunofluorescent labeling on paraformaldehyde-fixed brain tissues, followed by analysis with confocal laser scanning microscopy (Figure 3). IBA1-Gp positive microglial cells showed more than 94% colocalization with IBA1-Gt and P2Y12R-Rb markers in all examined developmental stages (Figure 3). The majority of cells lacking dual expression still exhibited the expression of at least one of the two proteins (Table 3). Based on this, both markers proved reliable for further microglia analysis. At E15, we observed that microglia were mostly restricted to the VZ and SVZ, with only rare presence in the cortical plate (Figure 3A). From P1 onward, the presence of microglia began to increase in the cortex (Figure 3E).

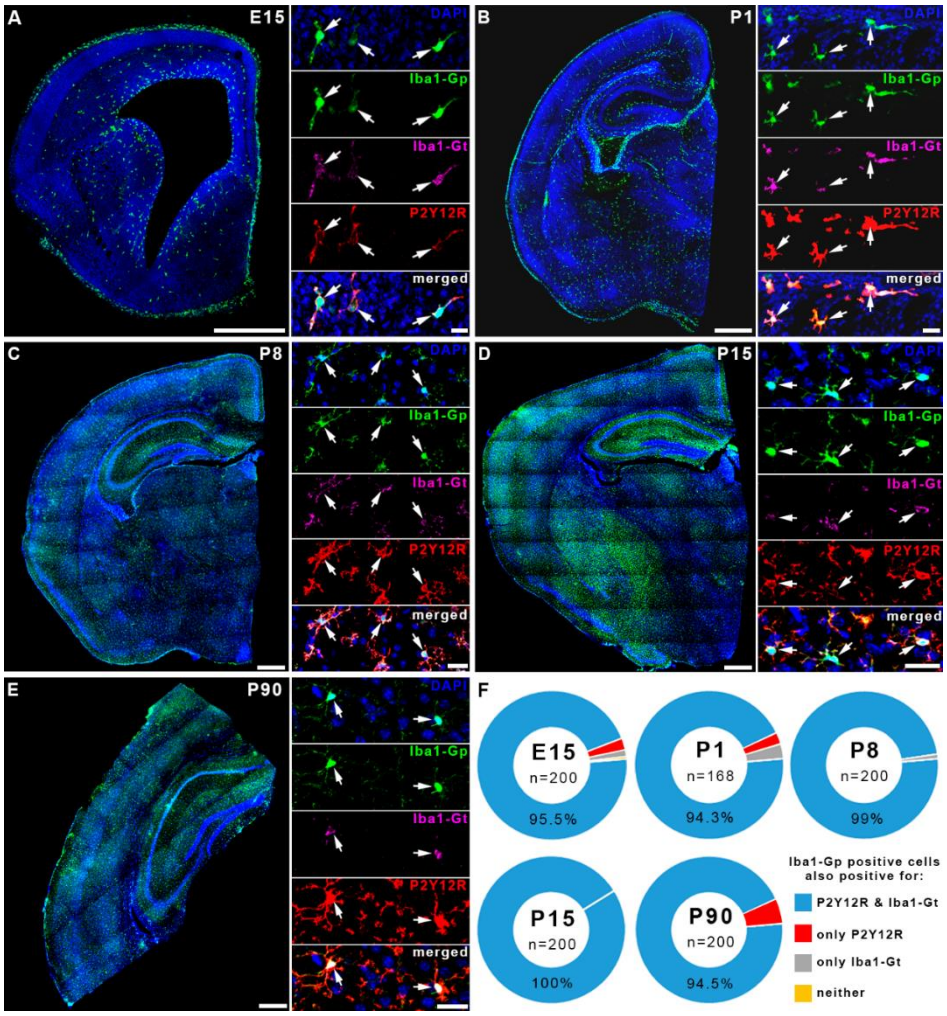

Figure 3. Microglial marker colocalization during brain development. A–E) Confocal images show IBA1+ microglia distribution in the mouse brain at E15 (A), P1 (B), P8 (C), P15 (D), and P90 (E). Insets highlight marker colocalization (IBA1-Gp: green, IBA1-Gt: magenta, P2Y12R: red, DAPI: blue), with white arrows marking microglial cell bodies. **F)** Most IBA1-Gp+ cells also expressed IBA1-Gt and P2Y12R. Insets/measurements were taken from the cortical plate (E15), neocortex (P1–P15), and hippocampal dentate gyrus (P90). Scale bars: 500 μm (large images), 25 μm (insets) (Adapted from 329).

To visualize neuroblasts and developing/immature postmitotic neurons, we performed doublecortin-immunolabeling (DCX) (334,335) (Figure 4). Within the DCX-positive pool, we did not distinguish between different maturation stages; therefore, these cells are collectively referred to as DCX-positive (DCX+) developing neurons in this study. We observed that microglial processes frequently contacted the cell bodies and neurites of DCX+ neurons during both developmental (Figure 4A) and adult neurogenesis

(Figure 4B). To exclude the possibility that these somatic junctions would be indeed interactions established with dendritic synapses, we tested whether interactions between glutamatergic synapses and microglial processes might occur near the cell bodies of developing neurons. We performed IBA1, DCX, and VGLUT1 labeling. At E15, we found no evidence of glutamatergic synapses (Figure 4C), nor were VGLUT1+ synapses detected in the granule cell layer of the DG (Figure 4D). However, combined labeling for IBA1, DCX, VGLUT1, and Homer1 revealed that microglial processes contact the synapses of both immature (DCX+; Figure 4E) and mature neurons (Figure 4F) in the molecular layer of the adult DG.

Our study builds on a previous discovery in which we identified a specific ultrastructural connection type between the cell bodies of mature neurons and microglial processes in the adult nervous system, allowing microglia to monitor neuronal status. Based on this finding, we hypothesized that a similar somatic purinergic connection might exist in developing neurons, enabling microglia to regulate proper neuronal development. To test this, we analyzed the prevalence of contacts between microglial processes and 3D-reconstructed DCX+ cell bodies using CLSM-microscopy across multiple developmental stages. These findings are part of a colleague's dissertation, however they are of critical importance to our research, and referencing them is justified to facilitate a more comprehensive understanding of the topic (336). We identified somatic microglial contacts as early as E15, a stage when glutamatergic synapses were not yet present (Figure 4A). The frequency of somatic contacts progressively increased during development: over one-third of developing neurons showed such contacts at E15 and P1, rising to approximately two-thirds by P8, and nearly all DCX+ cells receiving somatic microglial input by P15. This pattern was also observed during adult neurogenesis in the dentate gyrus of P90 mice. These results suggest that microglial processes frequently approach the cell bodies of immature neurons within a micrometer range. However, to confirm the presence of direct membrane contacts, further investigations using nanometer-resolution electron microscopy are required.

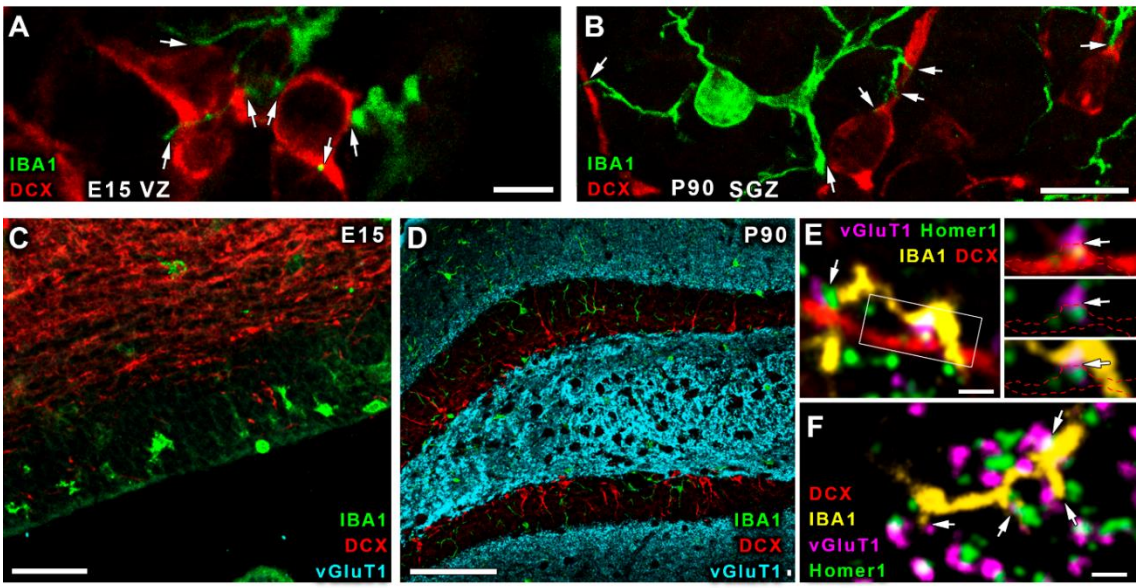

Figure 4. Microglial connections during developement. A–B) CLSM images show microglial contacts with immature cell bodies and neurites in the E15 ventricular zone (A) and P90 dentate gyrus (B). White arrows indicate contact sites. **C)** No synapses are present in the E15 cortical plate (IBA1: green, DCX: red, VGLUT1: cyan). **D)** Glutamatergic synapses are abundant outside the granule cell layer. **E)** Microglial processes (yellow) contact developing glutamatergic synapses, where VGLUT1 (magenta) and Homer1 (green) colocalize on a DCX+ dendrite (red) in the P90 dentate gyrus. White arrows mark the points of contact. Scale bars: 100 µm (D), 5 µm (A), 10 µm (B), 50 µm (C), 1 µm (E, F) (Adapted from 329).

4.1.2 Direct membrane-membrane contacts between microglia and cell bodies of DCX+ developing neurons

Based on light microscopy studies with micrometer resolution, we cannot definitively state that the numerous observed connections between microglial processes and DCX+ immature neurons are confirmed to be in nanometer proximity (136). To verify the presumed direct connections, we applied correlated light and electron microscopy (CLEM; Figures 5A–K). Through this method, we reconstructed 6 microglia-neuron contacts from the E15 VZ or SVZ and 10 contacts from the P90 dentate gyrus using serial sections. In all cases, direct membrane-membrane interactions between microglial processes and neuronal cell bodies were confirmed. Furthermore, CLEM revealed frequent enrichment of neuronal mitochondria at these contact points (Figures 5D, G–H), a hallmark morphological feature of somatic purinergic junctions (136). These

measurements also excluded the possibility that microglial processes would gather around the soma to contact perisomatic GABAergic-boutons.

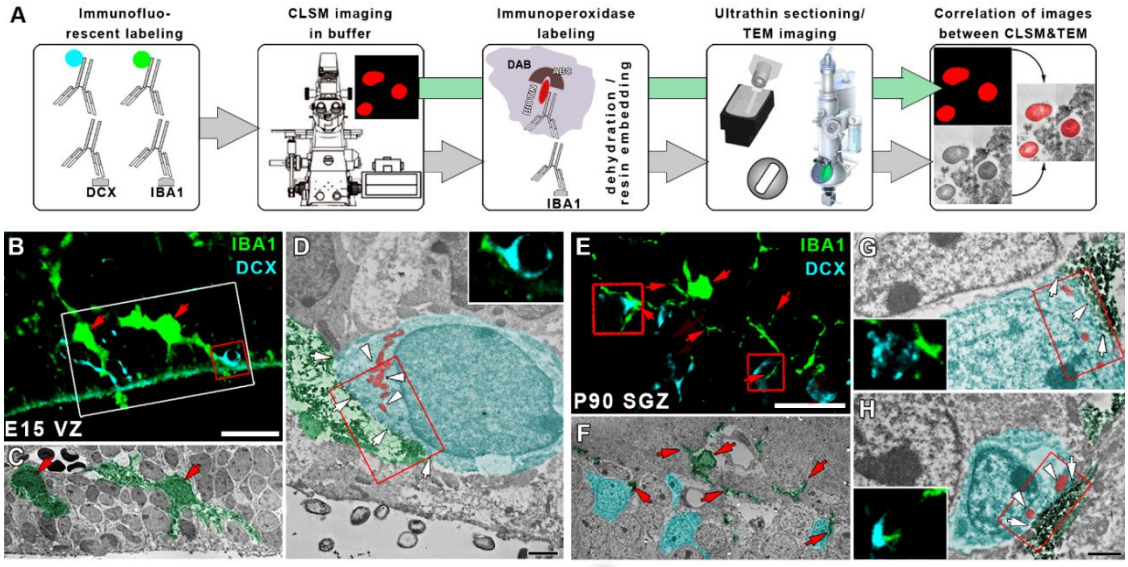

Figure 5. Microglial processes form direct membrane contacts with DCX+ immature neurons A) Schematic representation of the workflow combining correlated light and electron microscopy. Immunofluorescence labeling for IBA1 and DCX was performed, followed by CLSM imaging in buffer. The same sections were then processed for immunoperoxidase labeling, dehydrated, embedded in resin, ultrathin sectioned, and imaged using TEM. Correlations were made between images obtained from the two modalities. **B-D)** E15 mouse brain CLSM images show microglia-neuron somatic junctions (IBA1: green, DCX: cyan). TEM images confirm direct membrane-to-membrane contacts (white arrows) and neuronal mitochondria near junctions (white arrowheads). All six CLSM-identified contacts were validated by TEM. **E-H)** Similar analysis for a P90 mouse. The somatic junctions in the red boxes on (E) are enlarged in TEM images (G and H), with pseudo-coloring and annotations similar to the E15 analysis. All ten CLSM-identified contacts in this sample were confirmed as direct membrane-to-membrane contacts by TEM. Scale bars represent 30 µm (B); 4 µm (C); 1 µm (D), (G), and (H); 20 µm (E), 2 µm (F) ; this also applies to insets (Adapted from 329).

4.1.3 Components of the somatic connections of developing neurons

4.1.3.1 Mitochondria are enriched in somatic junctions at all stages of development

Mitochondria play a vital role in cells by powering cell activity, maintaining calcium balance, and regulating signaling, plasticity, and cell survival, ensuring optimal brain function and adaptability. In our earlier studies, CX3CR1+/GFP mice were electroporated *in utero* with the mitochondria-targeted CAG-Mito_R_Geco1 reporter construct (Figure 6A). We validated mitochondrial specificity of the construct in cell

cultures, confirming that the vector induced specifical mitochondria expression (Figure 6A). In adult mice, we observed the involvement of somatic mitochondria in microglial junctions (Figure 11B). Using *in vivo* two-photon (2P) imaging, we monitored the recruitment of microglial processes to neuronal mitochondria in the cerebral cortex of adult mice (Figure 6C). As anticipated, microglial processes were recruited to neuronal mitochondria, maintaining close apposition for a median duration of 29 minutes *in vivo* (n = 25 contacts on 19 neurons from 3 mice; Figure 6D). Additionally, CLSM analysis further confirmed the accumulation of neuronal mitochondria at somatic connections in the adult mouse cerebral cortex (136).

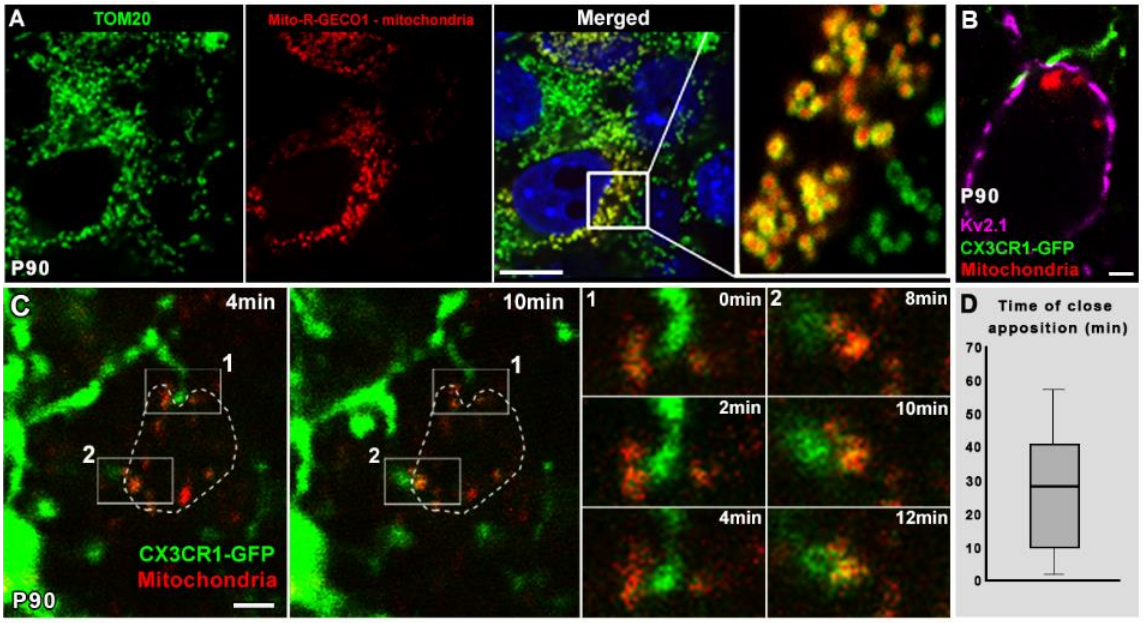

Figure 6. Mitochondria accumulate at the somatic junctions in mature neurons and persist for a long time. A) CMV-Mito-R-GECO1 construct was validated in transfected neurons, with TOM20 and DAPI counterstaining confirming specificity. **B)** The construct was also validated in perfusion-fixed brain tissue, revealing microglial processes (green) contacting neuronal somata near mitochondria labeled with Mito-R-GECO1 (red) at Kv2.1 clusters (magenta). **C)** In vivo 2P imaging (P90 CX3CR1+/GFP mice) showed microglial processes (green) touching mitochondria-enriched neuronal somata (dashed outline), with ROI enlargements illustrating somatic junction development. **D)** Contact lifetime was 29 min (median, 10-41 interquartile, n=25 contacts on 19 neurons from 3 mice). Scale bars represent 10 µm on (A; C), 4 µm on (B), (Adapted and modified from 21).

Based on these findings, we extended our investigation to examine the presence of neuronal mitochondria at somatic contacts on the cell bodies of immature neurons / neuronal progenitors. To quantify mitochondrial enrichment at these junctions, we utilized multiple immunolabeling techniques, correlated CLSM imaging, and a semi-

automated unbiased quantification method (Figures 7A–F, see *Methods 3.7.2.1.2.*). At all examined developmental stages, TOM20 (a mitochondrial marker) immunofluorescence intensity in the cell bodies and proximal tufts of DCX+ neurons was significantly higher at microglial contact sites compared to adjacent areas. Moreover, EM revealed that DCX+ neurons contacted by microglia displayed healthy chromatin structures (non-condensed) and normal mitochondrial morphology (Figures 7G). These findings confirm that developmental microglia-neuron somatic junctions are established between microglial processes and normal, non-apoptotic postmitotic neurons.

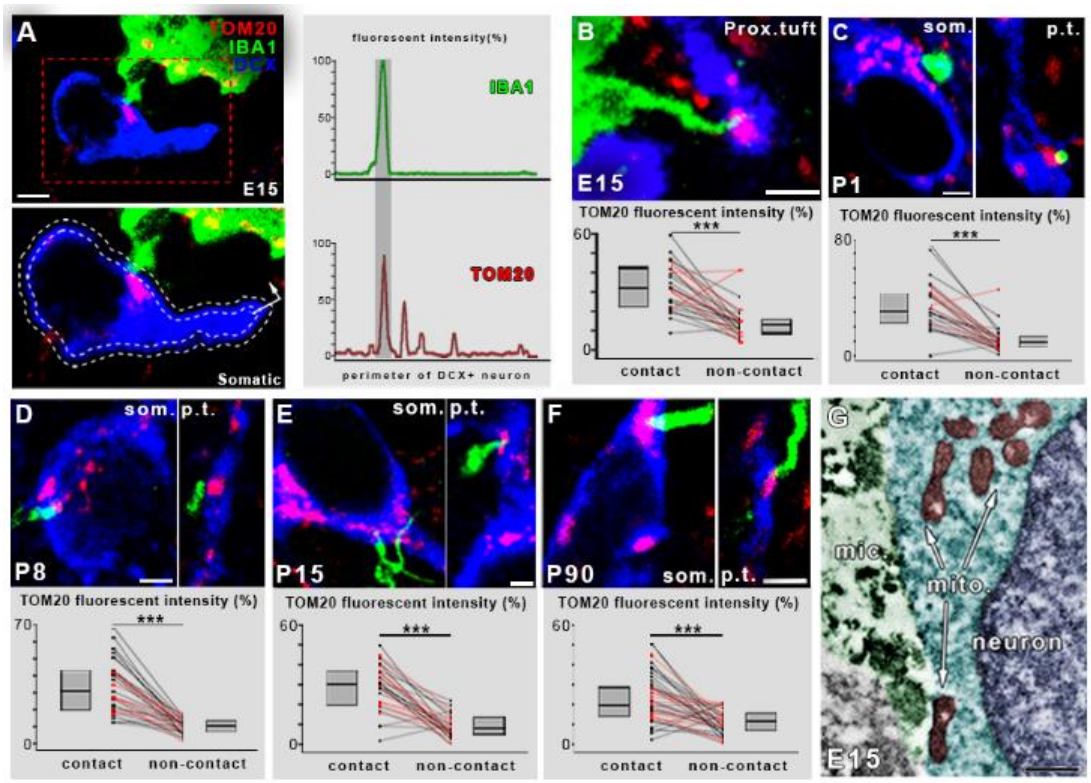

Figure 7. Mitochondria accumulate at somatic junctions in immature neurons. **A)** CLSM image shows an IBA1-labeled microglial process (green) contacting the cell body of a DCX+ immature neuron (blue) at a mitochondrion-rich site (TOM20, red). A semi-automated, unbiased analysis of fluorescence intensity across the perimeter of the neuron highlights significantly higher TOM20 intensity at the contact site. **B)** In E15 brains, microglia (green) contact the proximal tuft of DCX+ neurons, with TOM20 intensity significantly higher at contact sites. Data from 164 neurons (3 mice) show median and interquartile values (gray boxes).**C-F)** CLSM images from P1, P8, P15, and P90 mouse brains reveal mitochondrial enrichment at somatic and proximal tuft junctions in the cortical plate (E15), neocortex (P1–P15), and hippocampal dentate gyrus (P90) (3 mice/age). Wilcoxon test confirms higher mitochondrial fluorescence at junctions ($p < 0.001$). **G)** TEM image shows mitochondria with intact ultrastructure within somatic junctions. Scale bars: 500 nm (G), 3 µm (A), 2 µm (B–F). (Adapted from 329).

4.1.3.2 Lack of Kv2.1 proteins, and presence of lysosome accumulation in the somatic junction in developing neurons

Our previous studies revealed that somatic purinergic junctions are predominantly established at sites where neuronal exocytosis-promoting Kv2.1 clusters are located in the adult brain (136). Kv2.1 proteins regulate K+ fluxes (178) and form clusters with structural roles, anchoring the endoplasmic reticulum, promoting somatic exocytosis via SNARE interactions, and stabilizing transcellular contacts through adhesion proteins (179–181). To determine whether Kv2.1 accumulation is also a feature of somatic purinergic junctions during embryonic development, we performed immunofluorescence labeling on E15 and P90 mouse samples (Figures 8A, B). We found no Kv2.1 expression in the VZ/SVZ at E15 (Figure 8A), and although Kv2.1 was robustly expressed in mature granule cells in P90 dentate gyrus, none of the DCX+ cells expressed it (Figure 8B), suggesting alternative routes for microglial recognition of exocytosis sites at the neuronal cell body. In somatic microglial contacts of mature neurons, we observed that mitochondria-derived vesicles (MDVs) and cargo are often integrated into the endo-lysosomal pathway (337), with these vesicles positive for LAMP1 (136,338). Similar organelles were detected in DCX+ cell bodies near somatic contacts in E15 VZ/SVZ (Figure 8C) and P90 DG (Figure 8D). LAMP1+ puncta were found in 69% (E15) and 63% (P90) of examined somatic contacts (Figure 8E), indicating that cellular content may also be released at developmental somatic junctions from immature neurons.

cell body.

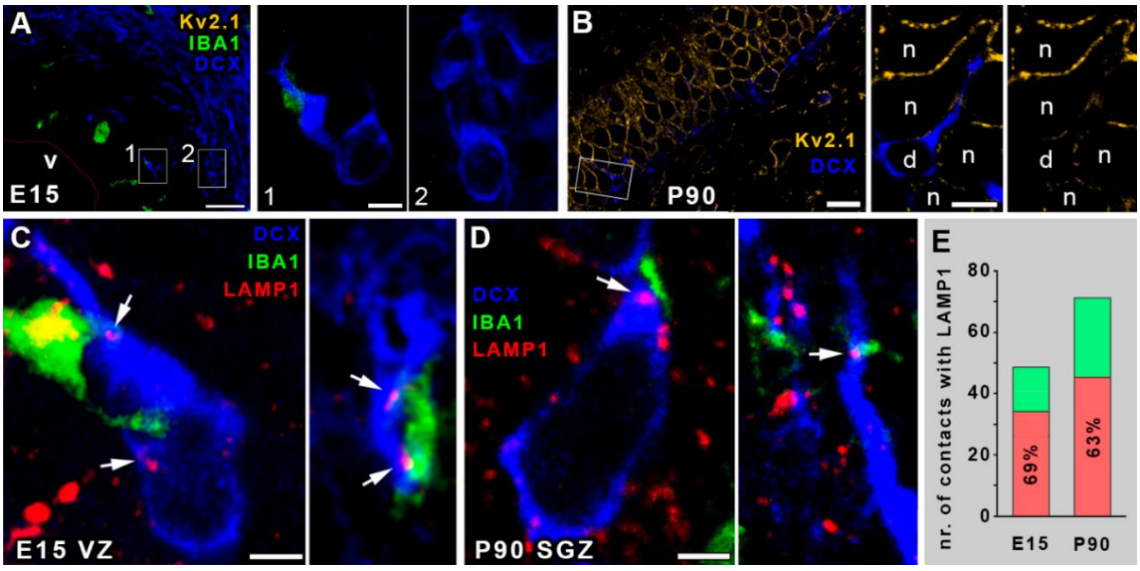

Figure 8. DCX+ developing neurons lack Kv2.1 but contain LAMP1+ lysosomes near somatic junctions. A) A CLSM image illustrates the complete absence of Kv2.1 (yellow) expression in the cortical plate of E15 mice. Among 142 fully reconstructed DCX+ cells analyzed from two mice, none exhibited Kv2.1 expression. DCX (blue) and IBA1 (green) are shown, with the ventricle (v) delineated by a thin red line. Enlargements of numbered rectangular areas are displayed on the right. **B)** P90 CLSM image confirms Kv2.1 expression in dentate granule cells but not in DCX+ neurons (136 cells, 2 mice). **C-D)** Microglial processes (green) contact DCX+ neurons (blue), with LAMP1+ lysosomes (red, white arrows) nearby (E15 SVZ/VZ, P90 DG). Images are from the SVZ/VZ of E15 mice (C) and the dentate gyrus (DG) of P90 mice (D). **E)** LAMP1+ vesicles present in 69% of contacts (E15) and 63% (P90) (n=49 for E15, n=71 for P90, 2 mice/group). The combined red and green columns represent the total number of measured contacts (n = 49 for E15, 71 for P90, with 2 mice per group), while red columns denote the subset of contacts with LAMP1 labeling (n = 34 for E15, 45 for P90). Scale bars: 30 μm (A), 15 μm (B), 2 μm (C, D) (Adapted from 329).

4.1.4 Somatic microglia-neuron communication with active, developing neurons is dynamic and P2Y12R-dependent

In our previous studies conducted in the cerebral cortex of adult mice, we observed the accumulation of the microglia-specific purinergic P2Y12 receptor at somatic connections. Through this receptor, microglia can detect ATP released by cells, directing their processes to the site of release and enabling the assessment of neuronal state. This receptor plays a crucial role in somatic connections, as several functions, such as neuroprotective effects and activity-dependent process coverage, are mediated in a P2Y12R-dependent manner (136). Due to its significance, we sought to explore the role of this receptor in somatic connections of developing cells. We observed contact-dependent accumulation of the receptor in all examined age groups, with detailed results presented in a colleague's dissertation. Subsequently, however, we wanted to reveal the dynamics of the somatic connection, for which we performed *ex vivo* 2-photon imaging of acute brain slices prepared from postnatal CX3CR1-GFP mice, loaded with the calcium-sensitive fluorescent dye Calbryte-590 AM (Cal- 590; Figures 9A and B). In cortical slices from P1 mice, we observed the dynamic formation of somatic junctions between microglial processes and the cell bodies of developing neurons (Figure 9C), resembling those previously identified in adult mice *in vivo* (136). The transient appearance, disappearance, and reappearance of these contacts indicate continuous, active communication between microglia and developing neurons, unrelated to cellular injury

or death. Given that microglia-specific P2Y12Rs are enriched on processes forming somatic junctions and are essential for junction formation and maintenance in the adult brain (136), we investigated the impact of acutely blocking these receptors on junction dynamics in samples from P8 mice. Following 15 minutes of baseline imaging, we applied either PSB-0739 (a selective P2Y12R inhibitor) or vehicle to the perfusion solution (Figure 9D). Dynamic somatic junctions continued to form between microglial processes and neuronal cell bodies (Figures 9E and G). During baseline imaging, the median lifetime of these contacts was 15 minutes, with 53% persisting throughout the observation period, consistent with data on mature somatic junctions *in vivo* (136). However, inhibition of P2Y12Rs caused a significant and rapid reduction in microglial coverage of developing neuronal cell bodies (Figures 9E and F), whereas the vehicle had no significant effect (Figures 9G and H). These findings indicate that P2Y12R somatic junctions. signaling is critical for the formation and maintenance of developing microglia-neuron.

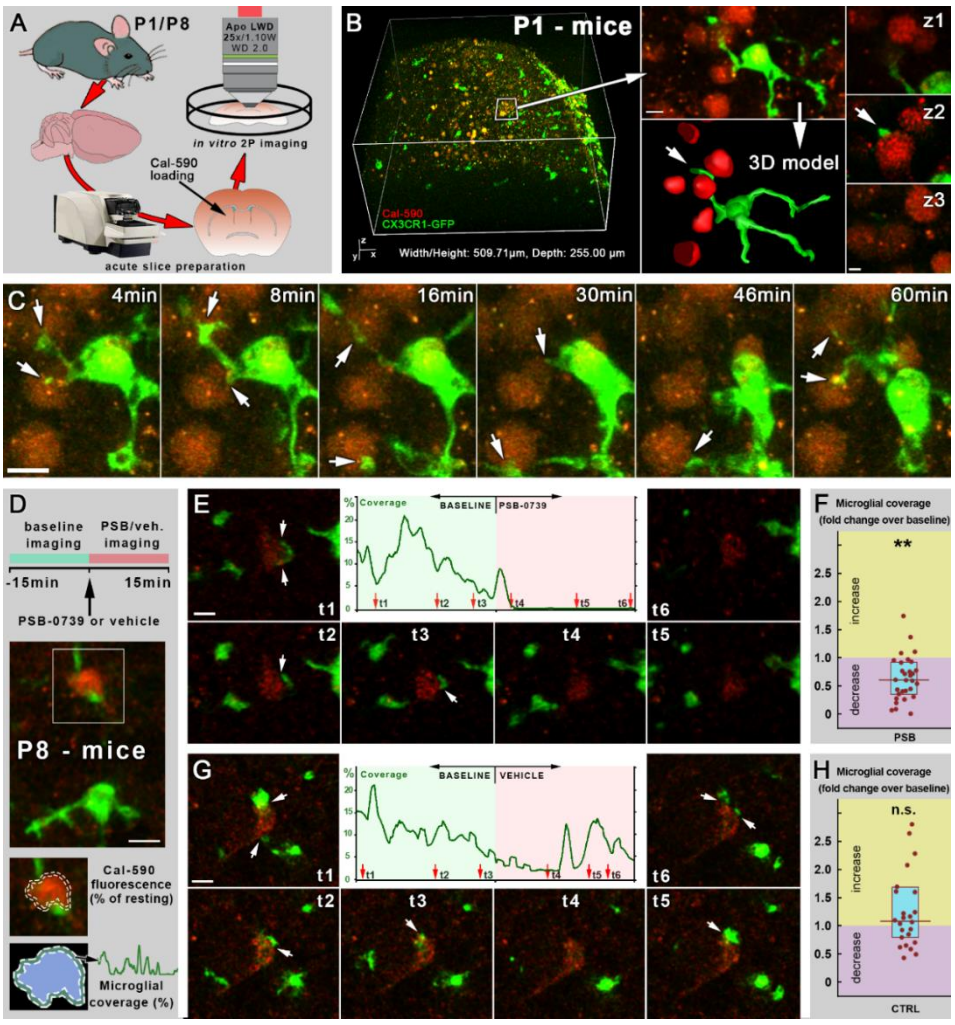

Figure 9. Microglia-neuron somatic junctions in developing neural circuits are dynamic and regulated by P2Y12R signaling. A) A schematic figure illustrates the experimental approach. **B)** wo-photon imaging (P1 neocortex) shows dynamic microglial-neuron contacts, with a magnified region and 3D model detailing a contact site (white arrow) which is visualized at the z2 plane, with z1 and z3 marking planes above and below the contact. **C)** Time-lapse imaging over one hour highlights the behavior of microglial processes (green, CX3CR1-GFP) forming transient somatic contacts with neuronal cell bodies (red, Cal-590). White arrows indicate microglial-neuron contacts, some of which are repeatedly established. **D)** Schematic figure of the P2Y12R inhibition experiment. **E)** P2Y12R inhibition ("PSB") reduced microglial process coverage on neurons, with calcium activity and contacts tracked over 30 min (white arrows, red arrows mark time points). **F)** Statistical analysis demonstrates that acute inhibition of microglial P2Y12Rs leads to a significant reduction in microglial process coverage on neurons (median 38% decrease from baseline; interquartile range 0.36–0.92; n = 30 cells from 3 mice). Coverage in the control condition is normalized to 1, and changes due to PSB are displayed as relative increases or decreases. Wilcoxon matched-pairs test; **p < 0.001. **G)** Control experiment using vehicle instead of PSB. **H)** Statistical analysis of vehicle treatment shows no significant change in microglial coverage (median 1.06-fold increase; interquartile range 0.75–1.66; n = 25 cells from 3 mice). Wilcoxon matched-pairs test. Scale bars: 10 μm (Adapted from 329).

4.1.5 Absence of P2Y12R signaling results in aberrant cortical distribution of DCX+ cells during development and leads to erratic cytoarchitecture in adulthood

To explore the impact of genetic P2Y12R deletion on postnatal neurodevelopment, we analyzed the density of DCX+ cells in cortical layers of wild-type (WT) and P2Y12R knockout (KO) mice at P8 (Figure 10). Ctip2 and Satb2 immunofluorescence staining delineated cortical layers (Figures 10B and C), and DCX+ cell density was measured in layers 6, 4/5, and 2/3 (Figures 10C–E). While no difference was observed in layer 6, P2Y12R KO mice exhibited significantly increased DCX+ cell density in layer 4/5 and a marked decrease in layer 2/3 compared with WT mice. This pattern indicates an abnormal cellular distribution in P2Y12R KO mice, with elevated density in layer 4/5 and reduced density in layer 2/3. Given previous evidence that the microglial receptor CX3CR1 is involved in developmental processes (143,229), we also examined whether CX3CR1 deletion might produce similar effects. However, CX3CR1 KO mice displayed no significant alterations in DCX+ cell density in layers 2/3 and 4/5, suggesting that P2Y12R deletion has a more pronounced and specific effect on DCX+ cell distribution. To assess

the functional consequences of P2Y12R deficiency on cortical cytoarchitecture in adulthood, we used high-resolution stereology on CLSM image stacks to measure neuronal density across cortical layers. In adult P2Y12R KO mice, we found significantly elevated neuronal density in layer 1, no differences in layers 2–5, and reduced neuronal density in layer 6 (Figures 10F and G). These findings highlight the critical role of P2Y12R-dependent microglial activity in regulating the proper postnatal distribution of DCX+ cells and establishing layer-specific neuronal densities in adulthood. The observed effects are likely mediated, at least in part, through developing somatic purinergic junctions, where P2Y12Rs are highly enriched and functionally pivotal.

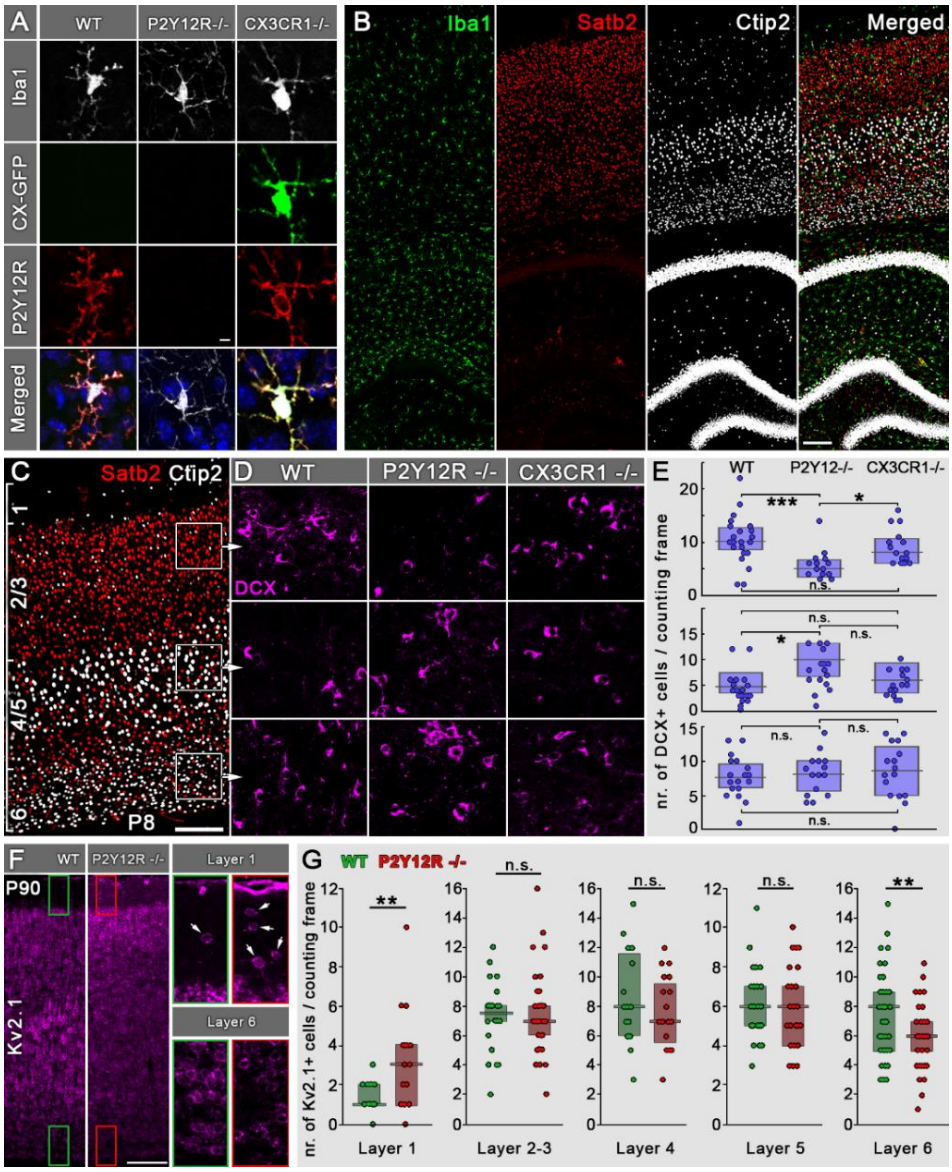

Figure 10. Microglial P2Y12Rs are crucial for proper cortical architecture A) CLSM images confirm genetic modifications using IBA1, GFP, and P2Y12R immunofluorescence, with DAPI-stained nuclei. **B)** P8 cortex CLSM images show IBA1, Ctip2, and Satb2 staining to define cortical layers. **C–E)** DCX+ cell density analysis showed no difference in layer 6 among genotypes (n=9). In layers 4/5, P2Y12R-/- mice had a 200% increase versus WT, while layers 2/3 showed a 50% reduction versus WT and CX3CR1-/- mice. Kruskal-Wallis test; *$p < 0.05$, **$p < 0.001$.**F-G)** Kv2.1 staining in adult neocortex: P2Y12R KO led to a 300% increase in neuronal density in layer 1 and 25% reduction in layer 6 (n=6, $p < 0.01$). Mann-Whitney U test; **$p < 0.01$. Scale bars: 5 μm in (A), 100 μm in (B) and (C), 20 μm in (D), 150 μm in (F), and 20 μm in insets (Adapted from 329).

4.1.6 Microglial P2Y12R signaling regulates formation of cortical cytoarchitecture by interfering with neuronal proliferation

To determine whether the observed cortical cytoarchitecture abnormalities result from impaired microglial regulation of neuronal precursor proliferation, we first examined the distribution of DCX+ cells at P1, a stage when this process is particularly active. In the deeper cortical layers near the SVZ, we observed that the absence of microglial P2Y12Rs significantly reduced the density of DCX+ cells (Figures 11A–D), confirming that microglial P2Y12R signaling plays a role in shaping cortical architecture as early as P1. We then assessed the potential impact of P2Y12R deletion on neuronal proliferation using the Ki67 marker (339) (Figure 11E). Analysis of Ki67+ cell density in the P1 cortex (Figure 11F) and the density of Ki67/DCX double-positive cells (a subpopulation of developing neurons shortly after division; Figure 11H; (340) revealed a significant reduction in both Ki67+ cells (Figure 11G) and Ki67/DCX double-positive cells (Figure 11I) in P2Y12R-deficient mice. These findings highlight the critical role of microglial P2Y12R signaling in regulating neuronal proliferation and early cortical development. It is not only postmitotic neuronal cells that can express the Ki67 proliferation marker, but also radial glial cells, which function as specialized neural stem cells in the developing brain, promoting the formation of neurons and glial cells (except microglia) while providing structural scaffolding for neuronal migration (341,342). Accordingly, we conducted a more detailed analysis of Ki67-positive cells using glial fibrillary acidic protein (GFAP) immunolabeling, as both radial glial cells and mature astrocytes express this marker (343–345). Almost none of the DCX-Ki67 double-positive cells showed GFAP expression (Figures 11I–J and L), and only a small fraction of Ki67+ cells (11%, interquartile range; Figures 11K and M) were GFAP-positive. This suggests that these

cells are not proliferating astrocytes but rather developing neurons, consistent with previous studies (334,340). These findings indicate that microglia regulate the proliferation of neuronal progenitors through P2Y12R-dependent mechanisms.

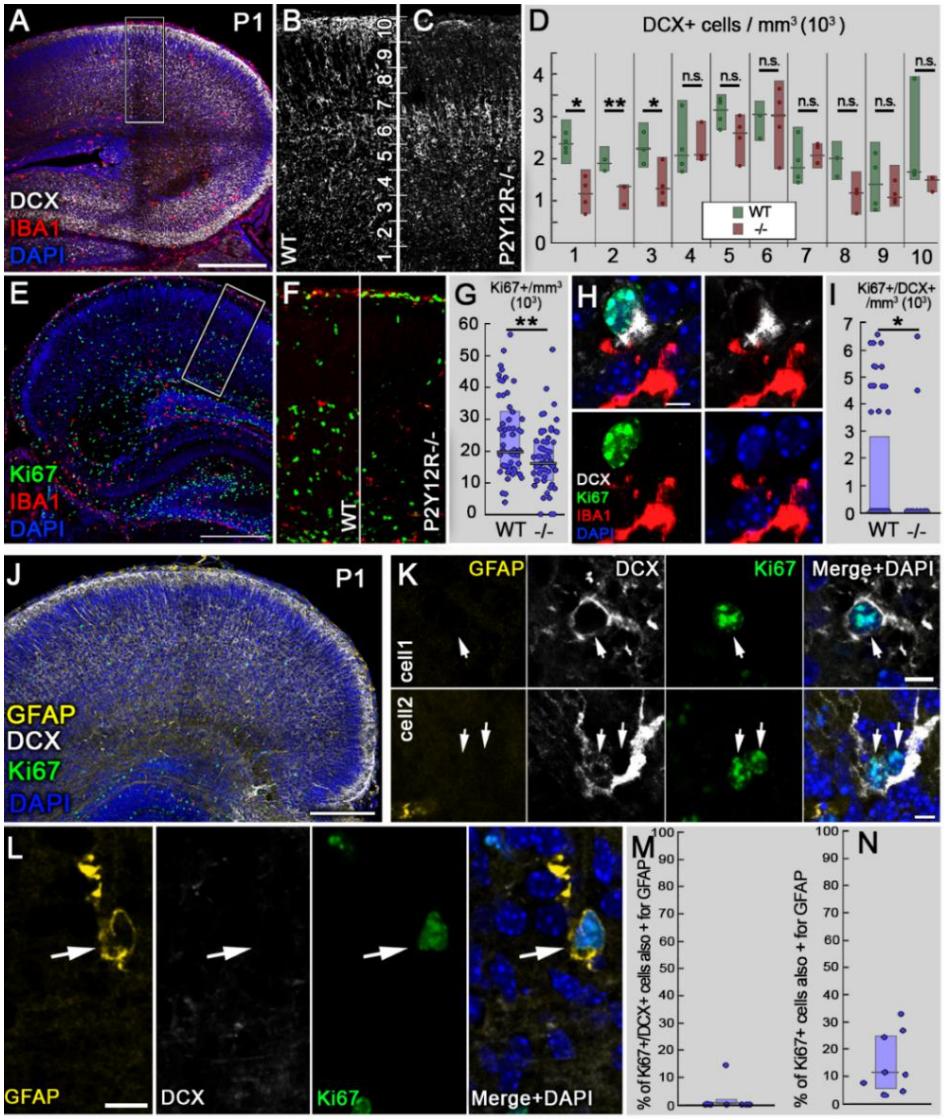

Figure 11. Microglial P2Y12R signaling regulates neuronal proliferation in the developing neocortex A–C) CLSM images (P1 mouse cortex) show DCX+ neurons (white), IBA1+ microglia (red), and DAPI-stained nuclei (blue), with a magnified region in (B, C). The cortex is divided into 10 zones from SVZ to the pial surface. **D)** P2Y12R deficiency reduces DCX+ cell density to 51% (zone 1), 72% (zone 2), and 58% (zone 3) of WT levels (*p < 0.05, *p < 0.01, n=6). **E–G)** Ki67+ proliferating cell density decreases to 82% of WT in P2Y12R-/- mice (p < 0.01, n=6). **H–I)** Ki67/DCX+ double-positive cells, marking proliferative activity, are absent in P2Y12R-/- mice (p < 0.05, n=6). **J–N)** GFAP/Ki67 analysis shows that Ki67/DCX+ cells lack GFAP, while only a subset of Ki67+ cells express GFAP (p < 0.01, n=3, Mann-Whitney U test). Scale bars: 300 µm (A, E), 5 µm (H, J), 200 µm (B, C, I), 150 µm (F), 20 µm (K). (Adapted from 329).

4.1.7 There is no detectable association between microglial regulation by P2Y12R and cell death

Given the frequent association between apoptosis and high proliferative activity during neurodevelopment, we explored whether the formation of somatic microglia-neuron junctions could be initiated by apoptotic cells. However, most DAPI-stained nuclei of DCX+ cells contacted by microglia exhibited healthy chromatin structures (Figures 12), while apoptotic cells were clearly identified by chromatin condensation, consistent with established markers (164). To confirm this, we utilized Annexin V immunofluorescence labeling (346) to detect postmitotic neurons that had exposed phosphatidylserine on their outer membrane leaflet, indicating commitment to apoptosis. A detailed, unbiased 3D analysis of high-resolution CLSM image stacks (Figures 12A–D) revealed that DCX+ cells with somatic microglial contacts were Annexin V-negative (Figures 12A), whereas Annexin V-positive cells displayed chromatin condensation (Figure 12A). These findings demonstrate that microglia contact viable developing neurons, and the frequent formation of somatic purinergic junctions on DCX+ cells is unrelated to phagocytosis of apoptotic cells. We also explored whether microglial P2Y12R signaling influences apoptosis in the developing cortex. The cortical density of Annexin V+ cells, Annexin V/DCX double-positive cells, and microglial contacts with Annexin V+ cells did not differ between WT and P2Y12R-/- mice (Figures 12B–D). These results suggest that cortical cell death at E15 and P1 does not differ significantly between genotypes. To further assess apoptosis, we used TUNEL labeling, a sensitive method for detecting DNA fragmentation associated with apoptosis (347). At E15 and P1, the absence of P2Y12R did not significantly affect the number of TUNEL-labeled puncta (Figures 12E–K), although a slight trend toward increased apoptosis in P2Y12R KO mice was observed at P1. Based on these results, it can be concluded that at the E15 and P1 stages, the regulation of apoptotic cells in the VZ, SVZ, intermediate zone (IZ), cortical plate (CP), and cortex (CX) regions does not occur in a P2Y12R-dependent manner. It is likely that this regulation takes place through other signaling pathways, in different brain regions, or perhaps at a later developmental stage.

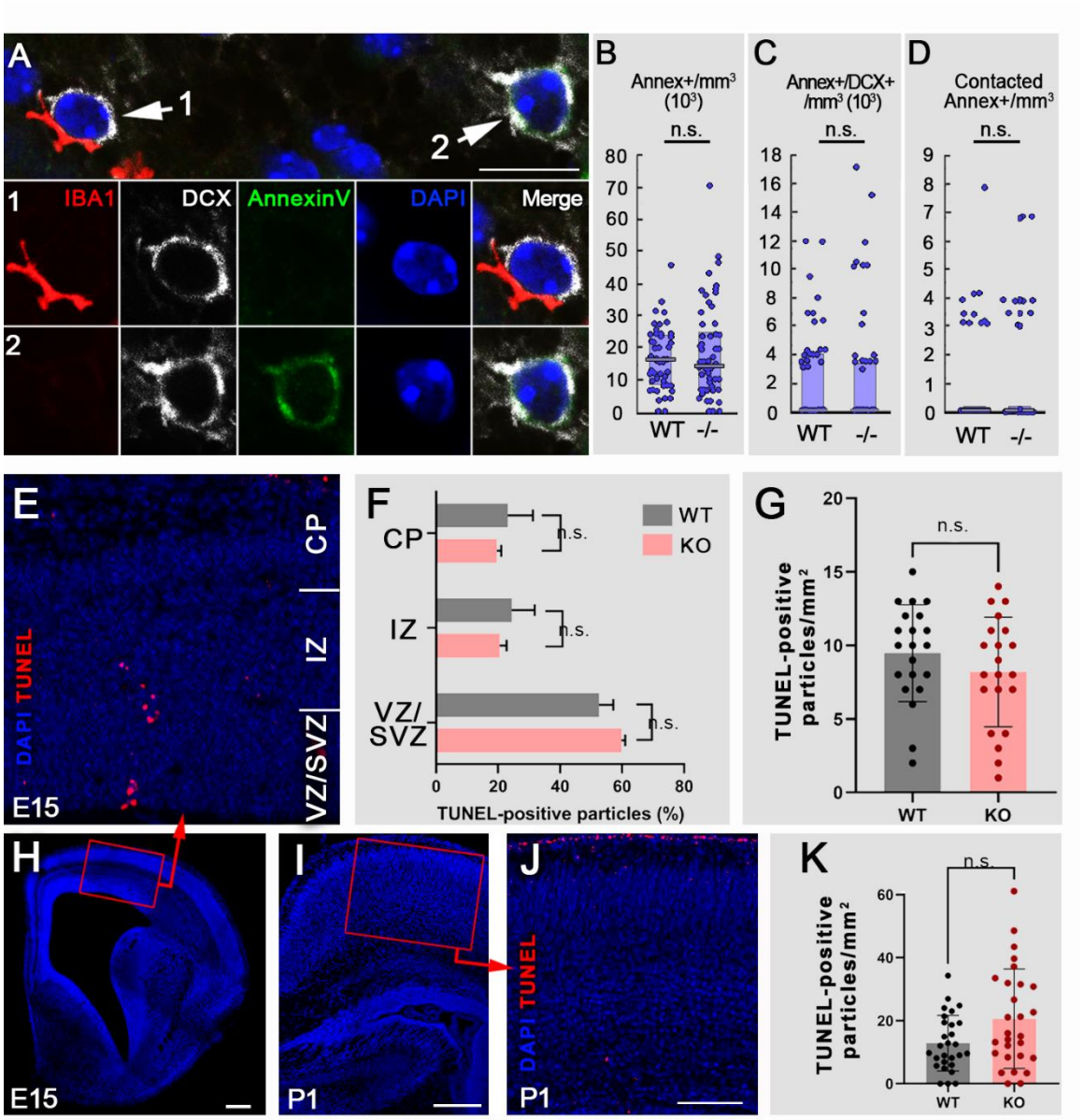

Figure 12. Microglial P2Y12R signaling and neuronal apoptosis A) CLSM image of immunofluorescent staining for IBA1 (red), DCX (white), Annexin V (green), and DAPI (blue), illustrating microglial contact with DCX+ cells and Annexin V/DCX+ cells without microglial interaction. **B-D)** Quantification of Annexin V+ and Annexin V/DCX+ cells in WT versus P2Y12R-/- mice (n = 6) with no significant differences (Mann-Whitney U test, n.s.). **E-G)** CLSM image of DAPI-TUNEL labeling in E15 cortex and quantification of TUNEL+ particles across cortical layers (n = 3 per group) with no significant difference (unpaired t-test, n.s.). **H-K)** CLSM images of DAPI-labeled E15/P1 mouse brains, highlighting measured areas. Quantification of TUNEL+ particles at P1 shows no difference between WT and KO mice (n = 3, unpaired t-test, n.s.). Scale bars represent 10 µm in (A), 200 µm on (H, I), 100 µm on (J, E) (Adapted from 329).

4.2 Altered microglial cell-cell interactions with ageing

Ageing affects not only the functioning of neurons but also fundamentally influences microglial cells. Ageing-related changes—such as reduced motility, impaired phagocytosis, disrupted proteostasis, telomere shortening, and chronic stimulation—collectively contribute to the decline in microglial functions (278). However, our understanding of contactology of microglial cells in this context is limited and the impact of altered cell-cell interactions on brain health and development of neurodegenerative diseases in the brain is unknown. To this end, we aimed to explore potential changes in the connectivity of microglial cells in the aged brain. First, we analyzed the neocortex of young adult (P90) and aged (P600) mice, followed by studying cortical regions from middle-aged and aged human (aged 55 - 60, and 79 - 85 years, respectively). Microglia were identified by the expression of the P2Y12 receptor (P2Y12R) and IBA1. To investigate their cell-cell interactions, we visualized different brain cell types: astrocytes with GFAP and AQP4, blood vessels with AQP4 and lectin, oligodendrocytes with CNP1, and neurons with immunohistochemical labeling against Kv2.1 (Figure 13), with a particular focus on somatic microglia-neuron interactions.

4.2.1 Microglial cell loss in ageing

Initially, we adopted a broader perspective to investigate how the ratio and the distribution of cells evolve and how these change with age. To achieve this, we employed multiple immunohistochemical labeling techniques and slide scanner microscopy (Figure 13). Using Z-stack images, we analyzed the distribution of cells throughout the full thickness of the cortical layer. Our study revealed a significant decrease in the number of microglia (Figure 13K) and neurons in the older age group, while an increase was observed in the number of oligodendrocytes (Figure 13I). In older samples, a marked heterogeneous distribution of microglia was particularly evident in the cortical regions (Figure 13K). Furthermore, a significant reduction in microglial cell numbers was also observed in human tissue samples derived from elderly patients (Figure 13J). These findings suggest that the balance of cells becomes disrupted with age, likely as a consequence of the detrimental regulatory mechanisms associated with ageing.

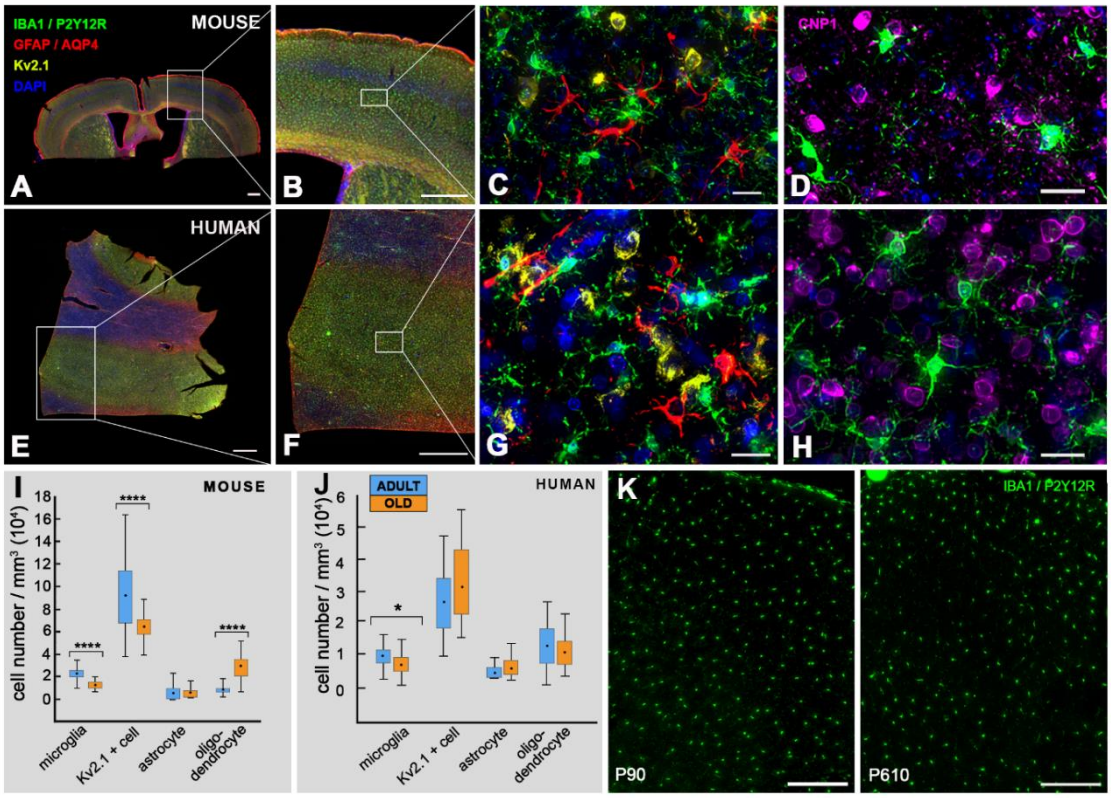

Figure 13. Changes in cell numbers with ageing. A-C, E-G) In adult mice and middle age old human samples, slide scanner images showing quadruple immunofluorescence staining for microglial cells (IBA1/P2Y12R in green), astrocytes and blood vessels (AQP4/GFAP in red), neurons (Kv2.1 in yellow), and nuclei (DAPI in blue). **D, H)** Oligodendrocytes visualized separately with CNP1 staining (magenta). These immunofluorescence stainings were employed to determine the density of cortical cell types. **I)** Microglial and neuronal cell numbers decreased in the aged group, while oligodendrocyte numbers increased (n = 4 mice). **J)** A decrease in microglial cell numbers was also observed in human samples (n = 4 patients). **K)** Overview images reveal the heterogeneous distribution of microglial cells. Median values and interquartile ranges are marked by boxes and whiskers. Blue boxes indicate P90 mice and human samples below 70 years of age, while orange boxes represent P610 mice and human samples above 70 years of age. Mann-Whitney U test; *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001. Scale bars: 500 µm in (A), 20 µm in (B, C, E and F), 1000 µm in (D), 200 µm in (I) (unpublished results).

4.2.2 Changes in microglial connections during ageing

To perform their highly complex and multifaceted functions, microglial cells must interact with all cell types in the brain. This continuous interaction enables them to sense the brain's current state and maintain its integrity. Extensive research in the literature supports the notion that microglial cells can connect with almost every cell type in the

brain (69). However, our knowledge about these interactions—particularly those involving non-neuronal cells—remains incomplete, especially regarding their frequency, spatial distribution, ultrastructural characteristics, signaling mechanisms, and functional significance. Moreover, the potential changes in these interactions during ageing are largely unexplored. Our research aimed to expand knowledge about the intercellular interactions of microglial cells and fill existing gaps, focusing on comparing adult and aged stages. To achieve this, we designed a fundamental anatomical study to map the number and types of connections a microglial cell establishes with different cell types at a given moment in both adult and aged states (Figure 14). We used the same immunolabeled samples for this study that were used for slide scanner imaging (Figures 14C-D, F-G). In confocal laser scanning microscopy (CLSM) images, we manually traced the processes of randomly selected microglial cells and analyzed their connection points with oligodendrocytes, astrocytes, neurons, and blood vessels (Figure 14A). We identified three main types of connections: process-to-process, soma-to-process, and soma-to-soma (referred to as satellite) connections (Figure 14B; Table 5). The most frequent type of connection was process-to-process interactions. Notably, in aged mice, we observed a significant decrease in process connections between microglia and astrocytes, which also showed a decreasing trend in human samples, but did not prove to be significant (Figure 14E, J; adult: median=12; aged: median=10). Conversely, in human samples, the number of process connections with oligodendrocytes significantly increased in older individuals, but no significant difference was observed in mice (Figure 19E; adult human: median=20; aged: median=26). Consistent with our previous slide scanner microscopy findings (Figure 14G), which showed an increase in the number of oligodendrocytes in older age groups, we found that the connections between microglial processes and oligodendrocyte somata also became more frequent in both mouse and human samples (Figures 14E, H; mouse and human: adult: median=0; aged=1). Satellite connections between microglial somata and the somata of other cells were extremely rare (Figures 14E, F), as expected given the noisier and slower communication typically associated with neuronal satellite connections (137). However, our results highlighted that oligodendrocyte processes often form at least one connection with microglial somata, underscoring the presence of bidirectional communication and emphasizing its significance (Figures 14E, H; mouse and human: median=2). Previous studies

demonstrated that microglial cells establish direct membrane contacts with all components of the neurovascular unit (NVU) (55). In this study, we did not differentiate between the various components of the NVU in their interactions with microglia. Instead, we examined microglial contacts identified through AQP4 labeling of astrocytic endfeet (Figures 14C, F). As in earlier observations, microglial cells were often located at the Y-branch points of blood vessels, with each microglial cell maintaining an average of 1–2 process connections with distinct vascular segments. In analyzing somatic connections involving neuronal cell bodies, we observed a significant reduction in aged samples for both mouse and human tissues (Figures 14E, J; mouse: adult: median=8; aged: median=6; human: adult: median=4; aged: median=3). We paid special attention to these somatic connections and analyzed their frequency from the neuronal perspective as well. Based on an analysis of randomly selected neurons, we found that neurons in aged samples had 20–25% fewer somatic connections (Figures 14N, O). Our findings suggest that the reduced frequency of somatic interactions mediated by microglia in older samples may lead to unregulated neuronal activity in the absence of microglial control. This could potentially contribute to ageing-related pathologies such as dementia.

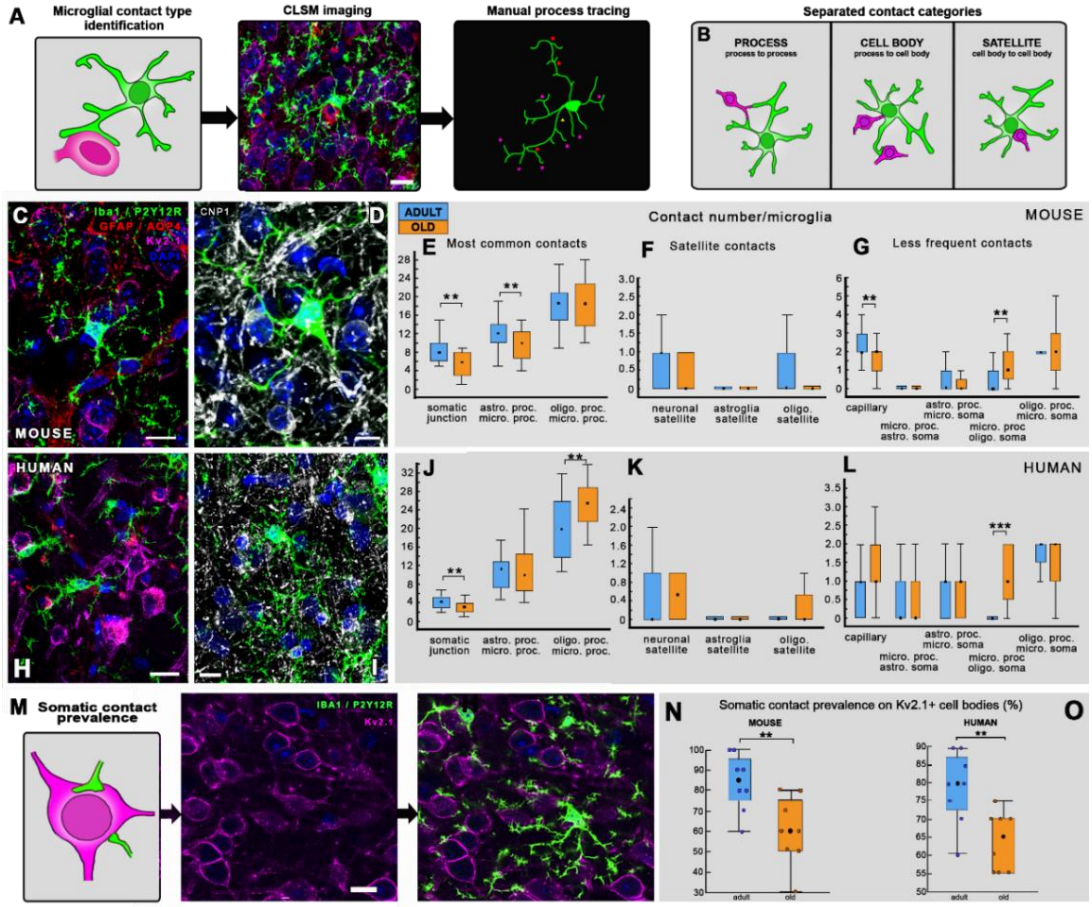

Figure 14. Prevalence of the somatic junction decreases during ageing. A, M) A schematic figure illustrates the analytical approach. **A)** The analysis examined the number and types of connections microglia established at a given time. **M)** The percentage of neurons with somatic connections was evaluated. **C–D, H–I)** CLSM images show quadruple immunofluorescence staining for microglia (green), astrocytes/blood vessels (red), neurons (yellow), and nuclei (blue), with CNP1-stained oligodendrocytes (white). Both mouse (C–G) and human (H–L) samples from adult and aged tissues were used in the experiment to characterize microglial connection types. **E–G, J-L)** Aging reduces microglial somatic junctions and astrocytic/capillary contacts while increasing microglial-oligodendrocyte soma interactions in both mice (n=4) and humans (n=4). **N–O)** Somatic junction prevalence significantly decreases with age (P90 versus P610 mice; <70 versus >70 years in humans, n = 4 patients; 4 mice; Mann-Whitney U test; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Scale bars: 10 µm (A, C, I, M), 5 µm (D), 20 µm (H) (Unpublished).

4.2.3 Possible compensatory mechanisms during ageing

In our previous studies, we observed that the surface area covered by microglial processes on neuronal cell bodies increased in response to neuronal activity and pathological states, such as stroke or COVID-19 (189,329). Based on these observations, we sought to determine whether a similar phenomenon occurs during ageing. For our investigation, we used IBA1, Kv2.1, and DAPI labeling and acquired CLSM z-stack images of the samples. In these images we measured the total surface area of neurons as well as the surface area contacted by microglial processes (Figures 15A, B, D, E). Our results showed that in aged samples, microglial process coverage doubled in both mouse (Figures 15B, C) and human samples (Figures 15E, F) (mouse: adult: median = 5%; old: median = 10%; human: adult: median = 2%; old: median = 4%). These findings suggest that during ageing, the increased coverage of neuronal surfaces by microglial processes may function as a compensatory mechanism to counterbalance the decline in the frequency of somatic connections, thereby contributing to the maintenance of neuronal homeostasis.

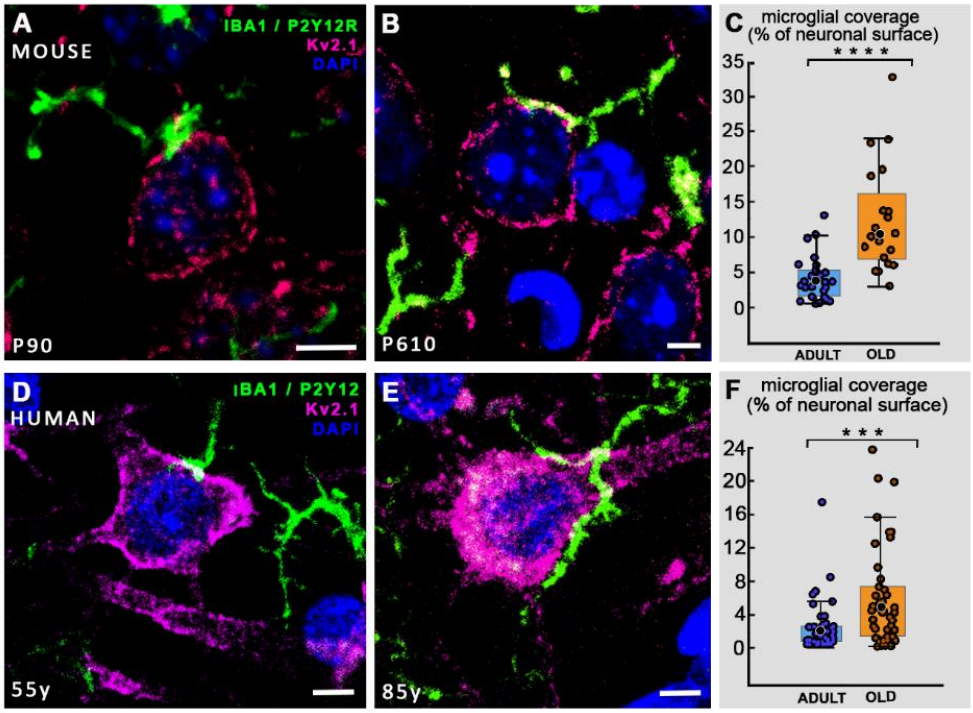

Figure 15. Increased process coverage on the cell body of nerve cells in older age. A–B, D–E) CLSM images show microglia (green), neurons (magenta), and nuclei (blue) in adult (P90) and aged (P610) mice, and human samples (55–60 versus 79–85 years old). **C, F)** Microglial coverage significantly increased with age in both mouse and human samples (n=4 mice, 4 patients; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; Mann-Whitney U test). Scale bars were 5 µm (A, B, D, E) (unpublished results).

4.2.4 Altered bidirectional communication between microglia and neurons during ageing

In addition to our earlier primarily anatomical studies, we conducted functional experiments to gain deeper insight into the communication between microglial cells and neurons. For this purpose, we developed an *in vivo* two-photon (2P) microscopy longitudinal measurement protocol, enabling us to study cell-cell interactions throughout the lifespan of mice. In our experiments, craniotomies were performed on mice at 50–60 days of age, followed by bi-monthly 2P imaging sessions (Figure 16B, C, D). During each measurement, we first created a map of the specific brain area to ensure the same cells could be revisited over time (Figure 16E, F, G). The experiments were conducted on anesthetized animals. Previous studies have shown that volatile anesthetics, such as isoflurane, can influence the motility of microglial processes (328). To address this, we

previously used 2P microscopy to evaluate the effects of different anesthetics on the motility of microglial processes (136) (Figure 16A). Our results revealed no significant differences between the anesthetics tested (median motility: isoflurane = 0.6 μm/min; fentanyl = 0.6 μm/min; urethane = 0.48 μm/min). Based on these results, we used fentanyl-based anesthesia, which showed a smaller deviation from 100% of the motility data. During the two-photon analysis, we examined neural events that exhibited at least a 20% increase in fluorescent intensity. We also analyzed the changes in fluorescence intensity (increases and decreases) of microglial contacts within a 1.5-minute interval before and after the calcium spike. As part of our research, we investigated the relationship between neuronal calcium activity and somatic connections. In adult specimens, we found that increases in tdTomato fluorescence intensity of microglial somatic contacts coincided with neuronal calcium spikes in 56% of cases. In contrast, in older animals, this coincidence dropped to only 21%. Our findings suggest that bidirectional communication between neurons and microglial cells significantly weakens with ageing, which may compromise the stability of nervous system function.

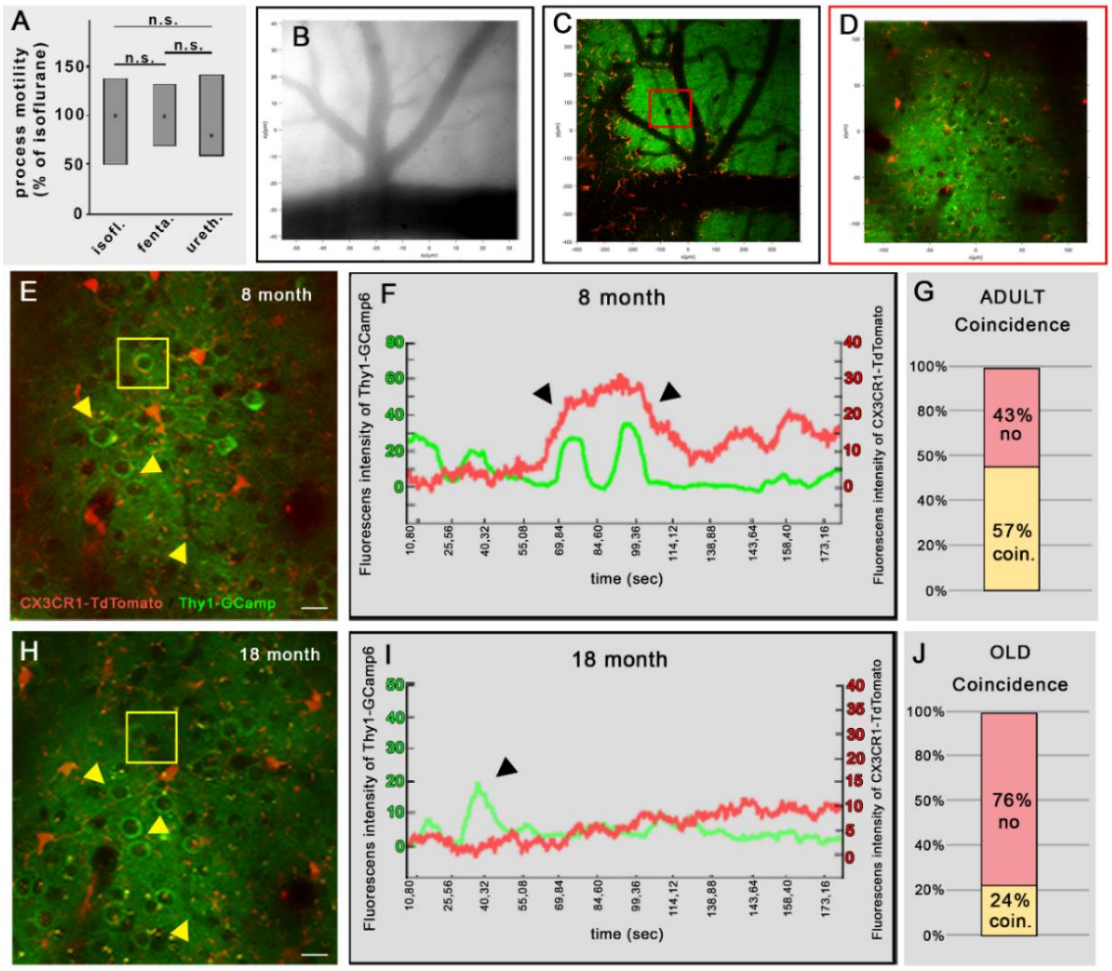

Figure 16. Impaired bidirectional communication between microglia and neurons with ageing. A) The result shows the effect of anaesthetics, in which no difference in microglial process motility was observed. Transgenic animals were used (Cx3CR1-TdTomato/Thy1-GCamp) in this series of experiments showing the neuronal calcium signal (green) and microglia (red). **B-D)** Schematic representation of the mapping for *in vivo* 2P longitudinal measurements to find the same cells throughout the lifetime of the mice. **E, H)** 2P image of the same mouse and cells at 8 and 18 months of age. Yellow squares indicate cells whose fluorescence values have been plotted. **F, I)** The curves show the change in fluorescence intensity of microglial somatic contacts (red) and neuronal calcium activity (green) over 18 min of measurement time. Black arrowheads shows calcium spikes. **G, J)** The frequency of mutual change between the two signals decreased in the older age group (n = 5 mice per groupe). Scale bars: 10 µm (A: Adapted from 21, unpublished results).

5. Discussion

Microglia, the primary immune cells of the CNS, play a vital role in cell development, function, and survival, ensuring brain homeostasis (348). These regulatory processes rely on close interactions with neurons, oligodendrocytes, astrocytes, and the vascular system (69,349). Microglia frequently connect with astrocytic and oligodendrocytic processes, while each cell in mice contacts an average of eight neuronal bodies simultaneously, emphasizing their key role in neuronal regulation (unpublished results). Neurons require continuous monitoring, which occurs in a compartment-specific manner (71,350,351). Well-documented synaptic regulatory function of microglia involves interactions with synaptic profiles, particularly during development (30,84,139,143,145,236–239,352,353). However, their role extends beyond synaptic regulation to neurogenesis, cell division, and differentiation (56,201,227,354). Since synapses form later, change dynamically, and are often distant from the cell body, microglial regulation likely centers on the neuronal soma, where integration of information and critical fate decisions occur.

Our previous research identified a distinct type of somatic connection in the adult CNS, characterized by direct membrane contacts and unique ultrastructure, independent of neuronal type (136). These junctions enable bidirectional communication via neuronal mitochondria and remain essential from development through aging (329) (unpublished results). Similar to adult neurons, developing neurons exhibit somatic connections containing mitochondria, purinergic vesicles, lysosomal compartments, and endoplasmic reticulum segments (329). While Kv2.1 and Kv2.2 proteins facilitate exocytosis in mature neurons, different proteins likely serve this function in immature neurons (136,329). These connections regulate cortical cytoarchitecture and neuronal proliferation through P2Y12R-dependent mechanisms (329). With aging, their number declines while microglial coverage increases, possibly due to weakened cell-cell communication (unpublished results). Aging-related microglial exhaustion may contribute to neurodegenerative disease development (104,278,285,355).

5.1 The role of the somatic connection in development

Microglia-neuron interactions at synaptic, dendritic, and axonal levels have previously been associated with various developmental processes (142,143,154,356–

358). However, these interactions alone cannot fully account for the wide range of microglial roles in development, as most cell fate decisions are closely tied to the neuronal soma (135,359,360). This highlights the importance of interactions between microglial processes and the somatic compartment of neurons. Microglial regulation of neuronal development begins early, in cells that have yet to form synapses. We propose that developing neurons, akin to mature neurons, establish somatic connections that enable microglia to effectively monitor their state and function (329). Evidence from the adult brain supports this, demonstrating that microglia engage in robust bidirectional communication that allows dynamic regulation of neuronal functions, cell fate decisions, and activity-dependent neuronal integration (136). Somatic connections with direct membrane contacts and specific ultrastructures have been identified on postmitotic neuronal somata (DCX-positive cells) during both early development and adult neurogenesis. As development progresses, the frequency of somatic connections increases, coinciding with a shift from depolarizing to hyperpolarizing GABA effects and profound changes in network activity patterns (329,336,361,362), suggesting that the emergence of more complex neuronal activity patterns requires a tighter microglial control.

Neuronal mitochondria play a crucial role in both developmental and adult neurogenesis, regulating differentiation and cell fate (363–369). Their structural dynamics guide neurogenesis, while they also respond to injuries, contribute to programmed cell death, and influence neuronal morphogenesis (370–374). At somatic purinergic junctions, mitochondria form a stable node that persists independently of microglia, suggesting an adaptive mechanism for ATP site recognition via P2Y12 receptors (136,375). These mitochondria serve as key microglial targets, regulating neuronal physiology and cell death in both developing and adult brains (136,167,376). Our research found a close association between somatic mitochondria in postmitotic cells and contacts formed by microglial processes during both developmental and adult neurogenesis, consistent with observations in mature neurons (136,329). Microglia are therefore ideally positioned at somatic contact points to sense signals from mitochondria, including purine metabolites (18,136,377,378), and regulate cellular functions. Through these connections, microglia can influence the activity or morphology of postmitotic cells by modulating their metabolism or mitochondrial function. Mitochondria-derived vesicles (MDVs) and other

mitochondrial components or signaling molecules released from neuronal somata carry critical information about the general state of the source cell (181,379). These released materials may include metabolic by-products, harmful proteins, mitochondrial metabolites, or even pathogenic agents (166–168,380,381), providing insights into the condition of the releasing neuron. Based on this information, microglia may intervene to maintain or restore neuronal homeostasis. It has been shown that the somatodendritic region of developing neurons is primarily responsible for exocytic events (382,383). In mature neurons, Kv2.1 channel clusters appear to play a major regulatory role in somatic exocytosis (384). While we did not detect the expression of Kv2.1 clusters in postmitotic cells, other delayed rectifier Kv proteins or alternative molecular scaffolds (385,386) may provide sufficient exocytic surface area.

Our findings, demonstrating the presence of LAMP1-positive lysosomes near the somatic junctions of postmitotic cells, suggest that cellular content may be released at these sites, serving as an optimal checkpoint for microglia. In our study, the vesicular nucleotide transporter, responsible for packaging purine nucleotides into vesicles, was functionally linked to ATP release through somatic junctions in mature neurons. The expression and colocalization of this transporter with LAMP1-positive vesicles were also observed in developing neurons (136,329,387). This indicates that purinergic nucleotides are key signaling candidates among the diverse range of molecules, including mitochondrial materials, potentially released at these junctions. Purinergic signaling and microglial P2Y12R have been shown to play essential roles in regulating neurogenesis and brain development (239,388). P2Y12R-mediated microglial signaling regulates neurogenesis, supports immature neuronal projections (389), and promotes the proliferation of subventricular zone (SVZ) cells in adult mice (390). Furthermore, microglia use P2Y12R signaling during development to couple phagocytosis with apoptosis progression (391). These findings underscore the importance of purinergic and P2Y12R-dependent pathways in microglial regulation of neuronal development. Robust P2Y12R expression has been observed in microglial processes involved in somatic junctions of mature neurons and also in developing neurons (136,329,336). These receptors are also expressed during human development (333), suggesting evolutionary conservation of microglia-neuron interactions. Since microglial processes at somatic junctions strongly express P2Y12R and are known to release purinergic metabolites in mature neurons (136), it is

likely that similar signaling pathways operate within these junctions to regulate neuronal development. Our *in vitro* two-photon microscopy results show that the formation and maintenance of somatic microglia-neuron junctions depend on P2Y12R signaling. Acute inhibition of this receptor caused a rapid reduction in microglial coverage of developing neuronal somata (329). This aligns with previous findings showing that the lifespan of somatic junctions significantly decreases following P2Y12R inhibition in adult mice *in vivo* (136)), indicating that similar communication pathways might be active during neuronal development. The dynamic formation and remodeling of these developmental somatic junctions suggest that microglia can closely monitor and influence neuronal activity through these specialized contact points. During neuronal development, shifts in activity patterns occur alongside the most active periods of synaptogenesis (392). In the first postnatal week, depolarizing GABAergic effects transition to hyperpolarization, glutamatergic synapses form, and primitive synchronized oscillatory activity evolves into mature activity patterns (361). By monitoring and regulating neuronal activity through somatic junctions, microglia may exert developmental control during this critical period, facilitating the establishment of complex neuronal networks. Supporting this idea, the prevalence of somatic junctions steeply increases during the first postnatal week, coinciding with these crucial developmental changes (329).

Several studies have highlighted the role of microglia in regulating neuronal migration during development, acting as specialized "guideposts" to facilitate this process (227,393). Some of these functions depend on CX3CR1, which is crucial for key developmental processes such as synaptic pruning, establishing proper neuronal connectivity, and ensuring the survival of layer 5 cortical neurons (143,229). However, our findings suggest that genetic deletion of P2Y12R in the developing neocortex affects processes distinct from those mediated by CX3CR1. P2Y12R deletion resulted in abnormal cortical distribution of postmitotic neurons at P1 and P8, whereas CX3CR1 deletion showed no such effect compared to controls. This indicates that somatic junctions, where P2Y12R is highly expressed, may serve as critical communication hubs through which microglia guide neuronal migration. Supporting this, we observed significant layer-specific changes in neuronal density in adult P2Y12R-deficient mice, with an increased density in layer 1 and decreased density in layer 6 compared to controls. We propose that these cytoarchitectural abnormalities may arise from disruptions in the

balance between neuronal proliferation and apoptosis. Our data on neuronal proliferation suggest that during cortical development, microglia regulate the proliferation of neuronal progenitors and potentially other cell types via P2Y12R signaling (329). Notably, DCX-Ki67 double-positive cells lacked GFAP expression, and only a small proportion of Ki67+ cells expressed GFAP, confirming that these cells are developing neurons rather than proliferating astrocytes, consistent with prior findings (334,340). Our findings align with earlier research demonstrating that the absence of P2Y12R in microglia reduces the density of DCX+ and Ki67+ cells in the adult dentate gyrus (244,329). Although we did not observe differences in apoptosis in P2Y12R-deficient mice, it is plausible that microglia influence cell death and the clearance of apoptotic neurons in the developing neocortex through P2Y12R-independent pathways or phagocytosis at different developmental stages (329).

In addition to its role in regulating microglial proliferation, microglia may also influence the migration of immature cells. It is possible that without proper microglia-neuron communication, neurons migrating during development through somatic connection may not receive adequate "stop" signals. This could lead to migratory "overshooting," resulting in a higher overall positioning of neuronal populations closer to the cortical surface. The differing direction of density changes observed in DCX+ cells at P8 compared to mature neurons in the adult brain may reflect alterations in the neuronal maturation process due to impaired microglia-neuron communication. Furthermore, the temporal absence of microglia between embryonic days E15 and E16 in the cortical plate, as described previously (394), could explain why density changes in the adult cortex are confined to layers 1 and 6, affecting the first and last waves of pyramidal cells. Alternatively, this phenomenon may result from the uneven subdivision of the preplate, which separates the marginal zone (future layer 1) and the subplate (future layer 6b). The precise mechanisms by which P2Y12R-mediated effects influence developing neural networks and adult cytoarchitecture require further investigation. Our observations align with prior studies implicating purinergic signaling in regulating neurogenesis. However, those findings primarily focused on adult neurogenesis in the dentate gyrus and the olfactory bulb (395,396). These mechanisms could also play a role in complex pathological conditions such as seizure-induced adult neurogenesis (389). Our current findings highlight the critical role of P2Y12R-dependent direct, cell-to-cell interactions

between microglia and the somatic compartments of developing neurons in physiological neurodevelopment. This reinforces the proposed broad significance of somatic purinergic connections in both developmental and adult contexts (136,329).

5.2 Microglial cells in ageing CNS

Microglia, the resident immune cells of the brain, play a pivotal role in maintaining the health of the CNS and responding to pathological injuries. Their role in neurodegenerative diseases, particularly in age-related conditions, has been widely studied (252,397,398). Historically described as "resting" cells that become "activated" during injury or disease, microglia are now recognized as dynamic, heterogeneous entities that continuously monitor their environment to preserve brain integrity (17,22,26,277). Ageing significantly affects microglial function, characterized by reduced motility, impaired phagocytosis, disrupted proteostasis and morphology, and altered intercellular interactions (278). Their phagocytic efficiency — critical for the clearance of cellular debris, amyloid-beta plaques, and myelin — declines, leading to the accumulation of harmful substances and synaptic waste (311,312). This loss of function is accompanied by a shift towards a pro-inflammatory state known as "inflammaging," characterized by increased baseline inflammation and excessive immune responses to stimuli (279). These changes are likely to undermine the ability of microglia to maintain homeostasis, increasing the brain's vulnerability to damage and disease (277,278). Based on this understanding, we explored the age-related changes in the microglial cellular network (contactology), an area with significant gaps in the literature.

Aging induces various changes in the cellular composition of the CNS, affecting neurons, microglia, astrocytes, and oligodendrocytes. During physiological ageing, the number of neurons remains stable in most regions, but functional and structural changes significantly impact neural connections and performance (399–404). Region-specific neuronal loss and synaptic alterations play a key role in age-related cognitive and motor decline (405,406). The number of astrocytes remains relatively stable during ageing, as supported by our findings, but their functionality undergoes significant changes (401,404,407). Alterations in gene expression, reduced metabolic efficiency, and a propensity for reactive states in ageing astrocytes weaken homeostasis, neuronal support, and blood-brain barrier regulation, leading to increased inflammation and reduced neuroprotection, which affect

brain health and ageing-related diseases (401,404,408). The total number of mature oligodendrocytes generally remains stable across many brain regions with ageing, although conflicting evidence exists in the literature (407–410). In our research, we observed an increase in the number of oligodendrocyte cells in aged mice and human samples. However, their ability to construct and maintain myelin diminishes with age, resulting in thinner, less compact myelin and white matter degeneration, contributing to cognitive decline and age-related CNS changes (411,412). Ageing also affects microglial function and morphology, leading to increased heterogeneity in their distribution and activity (278,280). While some studies report a decrease in microglial numbers in certain brain regions with age (278,311,413–415), others suggest stable or even increasing numbers (104,416,417), indicating that microglial changes are complex and region-specific (407). In our findings, we observed a reduction in microglial cell numbers accompanied by heterogenity in their distribution across the cortical regions of mice and the human neocortex. However, it is important to note that discrepancies in cell number data across the literature may stem from differences in immunohistochemical labeling, hence marker expression, with age, variations due to heterogeneity in cell state, and differences in the measurement methods used.

In the ageing brain, microglia exhibit reduced motility of their processes and diminished environmental surveillance capabilities, which also affect interactions between microglia and other brain cells (104,278,413). Microglia primarily communicate with astrocytes through their processes, with direct soma-to-soma contacts being very rare (55,62,112). In contrast, connections with oligodendrocytes, including both process-to-process and soma-to-soma interactions (98,100,102), are significantly more frequent and show an increased prevalence in older age. This finding aligns with the observed increase in the number of oligodendrocyte cells during ageing. The communication between microglia and oligodendrocytes plays a crucial role throughout life, as their mutual regulation is essential for maintaining the proper functioning of neurons (418,419). Microglia are responsible for regulating the proliferation of oligodendrocytes, and a disruption of this balance can lead to excessive oligodendrocyte proliferation (418,420). This phenomenon may also represent a compensatory mechanism triggered by myelin degradation and the ageing of oligodendrocytes. Changes in such interactions are significant in neurodegenerative diseases, such as Alzheimer’s disease and multiple sclerosis, where

myelin damage and oligodendrocyte dysfunction are central issues (98,419,421,422). The connections between microglia and neurons, particularly synaptic interactions, are extensively documented in the literature (142,143). A reduction in these connections can impair synaptic pruning and maintenance, contributing to synaptic dysfunction and cognitive decline commonly observed with ageing (405,406). This phenomenon is of particular interest in neurodegenerative diseases, such as Alzheimer's and Parkinson's disease (217,283,284,291,423–425). However, little is known about changes in somatic interactions, which are more likely to play a critical role in ensuring the healthy functioning and survival of neurons. Our findings reveal that microglia, on average, monitor eight mature neurons in mice and four in humans at any given time. However, the number of these interactions significantly decreases with advancing age. This decline may slow down the surveillance functions of microglia, endangered the maintenance of CNS homeostasis and their ability to respond effectively to injuries or pathological challenges.

An increasing number of studies have observed a P2Y12R-dependent increase in microglial process coverage on neuronal cell bodies, both under physiological conditions as a response to activity and in pathological states such as stroke and COVID-19 (136,189,329). In our research, we also noted an enhanced microglial process coverage in older individuals, suggesting that in the communication between the two cell types, neurons may require more intervention, facilitated by the expanded contact surface of microglia. One possible explanation is that somatic junctions not only play a role in restoring the physiological balance of neurons but also have a fundamental role in identifying severely damaged cells. In critical cases, microglia can initiate the phagocytosis of damaged cells through these contact points. This hypothesis is supported by a recent discovery indicating that the phagocytosis of dying neurons occurs in a coordinated manner: microglia are responsible for removing cell bodies, while astrocytes engulf neuronal processes (62). Another possible explanation is that organelle exchange may occur through the extensive contact surface, as has been suggested in other cell types under pathological conditions (426,427-433). This type of interaction could open new dimensions in understanding the communication between microglia and neurons, particularly in physiological and pathological contexts.

In addition we used precisely calibrated longitudinal *in vivo* 2P microscopy to examine microglia-neuron communication throughout the lifespan of mice. Our results showed that in adult mice, microglial process coverage of neuronal somata responded to changes in neuronal calcium activity in 56% of cases. However, this interaction decreased to 21% in older mice, suggesting that bidirectional communication between microglia and neurons declines with ageing, potentially destabilizing nervous system function. With ageing, the regulation of neuronal calcium activity deteriorates significantly, characterized by elevated intracellular calcium levels, disrupted calcium signaling, and impaired homeostasis (434–436). These changes could arise from mitochondrial and endoplasmic reticulum dysfunction (437), altered calcium channel activity (438), and reduced efficiency of calcium-buffering mechanisms (435). These disruptions can lead to neuronal hyperactivity, excitotoxicity (439), and reduced synaptic plasticity (440), all of which contribute to cognitive decline and neurodegeneration (441). Microglia are tasked with regulating these processes via somatic junctions, but ageing-associated microglial exhaustion may impair their ability to fulfill these regulatory roles, exacerbating calcium homeostasis disruptions. Significant changes also occur in microglial calcium activity during ageing. In young adult animals, microglia exhibit low-frequency spontaneous calcium transients that are critical for their surveillance and response functions. With ageing, these calcium signaling patterns change significantly: the proportion of microglia exhibiting spontaneous calcium transients peaks in middle-aged mice and declines in older mice (442,443). This suggests that ageing drives microglia into an exhausted, dysfunctional, and potentially senescent state. Age-related changes in microglial calcium signaling may impair their ability to interact effectively with neurons. Microglia rely on calcium signals to detect neuronal activity and respond to it, which is essential for maintaining CNS homeostasis (68). Altered calcium dynamics in ageing microglia may weaken their ability to regulate neuronal function, further promoting a pro-inflammatory "inflammaging" state and, in extreme cases, contributing to the development of neurodegenerative diseases. Understanding these age-related changes is crucial for developing interventions aimed at preserving microglial function and neuronal health during ageing. While the functional impairments of ageing microglia are significant, they do not necessarily lead to neurodegenerative diseases. Age-related changes are distinct but overlap with phenotypes observed in neurodegenerative conditions such as

Alzheimer’s disease. Additionally, factors such as chronic stimulation, systemic inflammation, genetic predisposition, or environmental influences can further exacerbate microglial dysfunction in ageing, accelerating the progression of neurodegenerative diseases (104,278).

5.3 Clinical relevance

One of the greatest unresolved challenges in modern medicine—posing a significant societal burden—is managing acute and chronic CNS disorders. Despite substantial infrastructural and financial investments, anticipated breakthroughs have largely failed to materialize A major reason may be the neuron-centric approach, even though understanding neurodegeneration requires examining all brain cell interactions (7–10). This underscores the need for alternative approaches that differ fundamentally from prior strategies for both acute injuries and chronic conditions. Insights from cancer research suggest that future therapeutic strategies may not rely on direct molecular intervention in complex pathologies but instead enhance the body’s natural defense and repair mechanisms through immune system fine-tuning (444,445). Developing targeted therapies is challenging due to the brain's isolation from peripheral circulation and immune processes by the BBB, limiting immune cell and protein access to neurons. Recent research, including ours, highlights microglia—key regulators of CNS inflammation—as a promising therapeutic target for neurodegenerative diseases (16,446–448). Microglia use dynamic, short-lived interactions to regulate neuronal function and maintain CNS homeostasis. Studies, including ours, show their crucial role in neuroprotection (55,73,136,162,189,329). In diseases like Alzheimer’s, Parkinson’s, stroke, epilepsy, and dementia, microglial dysfunction is among the earliest detectable changes, often preceding neuronal damage and symptoms (447–450).

Microglia colonize the brain early in development (203,205) and maintain a self-renewing population with dynamic gene expression changes throughout life (288,451,452). Their role in neurodevelopment, signaling pathways, and interactions is crucial, as even transient dysfunctions can cause lasting neuronal network disruptions (453–456). This underscores the importance of studying neuro-immune communication and microglia-neuron interactions, particularly in developmental disorders. Our experiments focused on postmitotic, immature neurons identified by DCX expression,

but microglial regulation is also active in embryonic stages, affecting neurogenesis, precursor proliferation, differentiation, migration, and survival (214,219,227–229). Microglial somatic interactions with dividing neuronal progenitors have been observed (219,457,458), but further research is needed to confirm their role as somatic junctions (329). Similarly, in vivo imaging studies are required to determine whether developmental phagocytosis (164,244,459) relies on somatic junctions as checkpoints. Mitochondrial changes are central to neuronal progenitor apoptosis, and microglia may detect and regulate these processes via programmed cell death and phagocytosis (460). The NAD+:NADH ratio is another key factor in stem cell fate regulation (365), and previous studies suggest microglia influence mitochondrial NADH levels via P2Y12R-dependent mechanisms (136). Further research should explore whether microglial morphological changes are driven by developing neurons' increasing demand for contacts or if they result from intrinsic microglial adaptations (452). Functional studies could identify neuronal content released at somatic junctions, the exocytic mechanisms involved, and how these processes evolve across neurodevelopmental stages. Additionally, understanding microglial quality control through somatic junctions (136) and its role in neural maturation is critical.

Microglia are uniquely suited to CNS protection due to their self-renewing capacity, high phagocytic activity, and metabolic flexibility (67,288,461). These traits allow them to support neuronal survival, even post-stroke, until their reserves are depleted—sometimes at the cost of self-sacrifice (136). A similar process occurs in infections (73) or apoptotic neuron clearance (164), where microglia remove debris to prevent inflammation (462). However, with aging, harmful substance accumulation and declining phagocytic activity may lead to microglial exhaustion (311,312). Rapid microglial replenishment is essential, as studies show that following CSF1R inhibitor-induced depletion, microglial numbers recover within two weeks (463). However, regenerative capacity declines with age. Interestingly, microglial repopulation in aged mice enhances hippocampal neurogenesis, synaptogenesis, and spatial learning (213). This repopulation originates from resident microglial cells that survive depletion (216), but if these cells carry genetic mutations, depletion therapy may not be effective. In such cases, potential treatments include donor-derived microglia transplantation or microglia differentiated from pluripotent stem cells.

Our research observed increased microglial process coverage on the surface of neurons associated with the presence of Kv2.1 clusters, which disintegrate through dephosphorylation under pathological conditions (136), leading to enhanced Kv2.1-dependent potassium (K+) efflux (178). This process plays a key role in the apoptotic cascade, which can be mitigated through pharmacological or genetic inhibition of Kv2.1 channels (429,464). Microglia fulfil a neuroprotective role by regulating extracellular K+ concentrations, exerting their effects by altering ion gradients or modulating Kv protein phosphorylation, which provides a foundation for developing neuroprotective therapies (428,465–467).

In common neurological diseases, neuronal mitochondrial damage is a key pathogenic factor, and evidence suggests that microglia-mediated processes could enable early detection of mitochondrial damage in neurons and targeted regulation of mitochondrial function (176). Strategies aimed at enhancing mitochondrial activity and reducing oxidative stress represent potential interventions to address age-related dysfunctions (468–470). Through the somatic junctions, microglia are ideally placed to influence these processes. Therefore, our goal is to identify proteins and molecular pathways that could serve as therapeutic targets for these conditions.

Strategies aimed at removing exhausted microglial cells, promoting the proliferation of new cells, enhancing proteostasis in existing cells, restoring phagocytic efficiency, and modulating inflammatory responses could alleviate age-related neurodegenerative conditions (278,295). By restoring the balance between protective and excessive microglial functions, these approaches have the potential to delay age-related cognitive decline and improve brain resilience. Future research should focus on elucidating the molecular pathways underlying microglial ageing to develop therapeutic interventions that preserve CNS integrity and function. Targeting the mechanisms of microglial ageing holds promise for maintaining CNS health and mitigating neurodegenerative diseases (278). I believe that a comprehensive understanding of microglial interactions with other cells could enable selective, microglia-specific interventions to help these "guardians" of the CNS, thereby making neurons more resilient to harmful insults affecting the nervous system. Therefore, our goal is to identify proteins and molecular pathways that allow us to selectively modulate microglial function for therapeutic purposes.

6. Conclusions

This thesis highlights the crucial role of microglia in maintaining CNS integrity through dynamic interactions with neurons and glial cells. As adaptive immune cells, microglia play essential roles from development to aging. Our findings emphasize the significance of microglial somatic junctions, which enable direct neuron-microglia communication, influencing cell fate, metabolism, and survival. During development, microglia regulate neuronal proliferation, migration, and cortical architecture through purinergic signaling, particularly P2Y12R-mediated pathways. However, with CNS aging, microglial function declines, marked by reduced motility, impaired phagocytosis, and pro-inflammatory shifts ("inflammaging"), contributing to cognitive decline and neurodegeneration. Aging also leads to a loss of somatic junctions, weakening microglial surveillance and neuron-microglia communication, increasing neuronal vulnerability to stressors. Additionally, microglia-glia interactions change with aging, particularly with oligodendrocytes and astrocytes, influencing CNS homeostasis. The rise in oligodendrocyte interactions may indicate compensatory mechanisms against myelin degradation, but also suggests shifts in glial communication dynamics requiring further research. Clinically, these insights call for a paradigm shift in neurodegenerative disease treatment, extending beyond neuron-centric approaches to broader CNS cellular interactions. Microglia present promising therapeutic targets, offering phagocytosis, metabolic flexibility, and dynamic signaling as intervention points. Enhancing microglial function—via phagocytic efficiency, proteostasis restoration, or inflammatory regulation—could mitigate age-related neurodegeneration and improve CNS resilience. In conclusion, microglia are central to CNS health, acting as key regulators from early development to aging. Understanding their interactions and regulatory roles will open avenues for innovative neuroprotective therapies, preserving CNS integrity and addressing the burden of neurodegenerative diseases.

7. Summary

Microglial cells, as indispensable immune cells of the central nervous system (CNS), play a crucial role in maintaining the integrity of the nervous system throughout life. Microglia interact dynamically and multifacetedly with almost every cell type in the brain, contributing to the regulation of CNS physiological function and providing protection against pathological insults. During development, microglia are among the first cells to appear in the CNS, regulating neuronal proliferation and migration, thereby shaping the proper cortical cytoarchitecture through somatic purinergic connections with immature neurons. This direct interaction, characterized by a highly specific ultrastructure, serves as a key mechanism of communication between microglia and developing neurons, enabling the monitoring and regulation of neuronal states while influencing mitochondrial function and metabolism.

With ageing, a decline in microglial functions becomes apparent, including reduced motility, impaired phagocytic activity, and increased baseline inflammation. This deterioration undermines the maintenance of neuronal homeostasis and contributes to cognitive decline as well as an elevated risk of neurodegenerative diseases, such as Alzheimer's and Parkinson's disease. Ageing disrupts the delicate balance of cell distribution and interactions, shedding light on broader processes associated with ageing. As age advances, a marked decrease in the frequency of somatic interactions occurs, coinciding with an increased neuronal need for support. To counterbalance this, an increase in microglial coverage of neuronal soma surfaces is observed, which supports neuronal health—an effect whose significance has been confirmed in conditions like stroke and infectious pathologies.

The central role of microglia in both developmental and ageing processes highlights the importance of supporting microglial functions. A deeper understanding of microglial interactions and regulatory mechanisms paves the way for innovative therapeutic strategies aimed at enhancing neuroprotection, preserving CNS integrity, and addressing the growing burden of neurodegenerative diseases.

8. References

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10. Acknowledgements

First and foremost, I would like to express my deepest gratitude and love to my parents, whose boundless selflessness, dedicated efforts, and loving support — both financial and emotional — have made it possible for me to freely discover my vocation. With heartfelt thanks, I also acknowledge my brother, who set an inspiring example for me and from whom I learned the values of perseverance, dedication, and the importance of creativity through the love of art. I owe immeasurable gratitude to my husband, who provided indispensable support by creating a warm and nurturing home and shouldering life's burdens, making the completion of this dissertation possible. I am profoundly grateful to Csaba Cserép, who took me under his wing from the very beginning and introduced me to the fascinating world of research. He not only taught me the nuances of the profession but also shared valuable life lessons, all with warmth and genuine friendship, for which I will always be deeply thankful. To Ádám Dénes, I extend my heartfelt thanks for supporting my ambitions alongside my sports career from the start and encouraging me to find my place in the field of research. The freedom he provided for my research has been a constant source of inspiration, and his openness and ever-present support are qualities every researcher aspires to find, for which I am truly grateful. I would also like to express my heartfelt gratitude to Rebeka Fekete for introducing me to the world of *in vivo* methods and for her patience and support in helping me master the intricate details and refined techniques of various surgical procedures. Finally, but no less importantly, I wish to thank all the members of the Neuroimmunology Research Group, especially Eszter Szabadits and Balázs Pósfai, for fostering an exceptionally inspiring and friendly atmosphere in which to work, as well as Zsolt Lele for his invaluable guidance in mastering *in vivo* electroporation techniques.