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Microglia-precursor cell interactions in health and in pathology

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Abstract: Until recently, microglia were mainly known as the resident phagocytes of the brain, i.e. the 'immunological warriors' of the brain. However, extensive knowledge is being accumulated about the functions of microglia beyond immunity. Nowadays, it is well accepted that microglial cells are highly dynamic and responsive, and that they intervene in a dual manner in many developmental processes that shape the central nervous system, including neurogenesis, gliogenesis, spatial patterning, synaptic formation and elimination, and neural circuit establishment and maturation. The differentiation and the pool of precursor cells were also shown to be under microglia regulation via bidirectional communication. In this concise review, I discuss our recent work in microglia-Pax6^{+} cell interactions in one of the circumventricular organs, the pineal gland. An analogy with the rest of the central nervous system is also presented. In addition, I briefly examine mechanisms of interaction between microglia and non-microglial cells in both health and disease. New avenues are also introduced, which may lead us to better comprehend the impact of microglia in physiological and pathological conditions.

Microglia are the residential phagocytic cells of the central nervous system (CNS). Since their first characterization, microglial cells have been associated with defense mechanisms against invading microbes or insults, and with processes of injury resolution (Tambuyzer *et al.*, 2009; Wake *et al.*, 2013). However, our understanding about how microglia function in both health and pathologies has been exponentially evolving. Nowadays, it is well accepted that microglia also contribute to the normal development and homeostasis of the CNS, which results in modulation of behavior (Tay *et al.*, 2017b). Precisely, microglia have been related to a broad spectrum of biological phenomena that shape the CNS, including regulation of cell populations and spatial patterning, myelination, and establishment and refinement of neuronal wiring by facilitating axon growth and synaptogenesis and executing synaptic pruning (Frost and Schafer, 2016; Arcuri *et al.*, 2017).

The embryonic origin of the microglial cells has been a controversial topic for decades. It is important to note that microglia do not originate from progenitors in the neural tube, as do neurons and the primary glial cells (astrocytes and oligodendrocytes) (Darnell and Gilbert, 2017). Instead, cutting-edge technology, including cell-specific targeting, *in vivo* imaging, and gene expression profiling, has recently shown that microglia derive from outer myeloid precursor cells, which are present in the embryonic yolk sack (Tay *et al.*, 2016; Prinz *et al.*, 2017). Then, the microglial precursors migrate and colonize the developing CNS before differentiation of the main cell types has commenced. After establishment, microglia become long-lived parenchymal residents, and they apparently maintain themselves by stochastic self-renewal during steady-state conditions or by clonal expansion in adverse environments (Tay *et al.*, 2017a).

Conventional research attempted to categorize the microglia phenotype, based on morphology or functional state. A ramified cell with multiple fine processes was pointed as a 'resting' (i.e. passive) microglial cell, while an amoeboid-like cell has long been accepted as an 'activated' microglial cell (Kettenmann *et al.*, 2011). In addition, microglia were divided based on their cytokine profiles into 'classically activated' M1 pro-inflammatory or 'alternatively activated' M2 anti-inflammatory cells (Kigerl *et al.*, 2009). However, these quite rigid and simplifying definitions have been replaced by the concept that microglia are constantly active, and highly dynamic and plastic cells in terms of both shape and phenotype (Raivich, 2005; Ransohoff, 2016). Moreover, microglial features and microglia-mediated regulatory circuits vary with the stage of development, the location within the CNS, the sex, and the nature and intensity of stimuli (Gordon *et al.*, 2014; Grabert et al., 2016; Matcovitch-Natan *et al.*, 2016; Tay *et al.*, 2017a; Thion *et al.*, 2018). Transcriptomic analyses have also revealed how microglia's agile behavior helps them to surveil and respond to their

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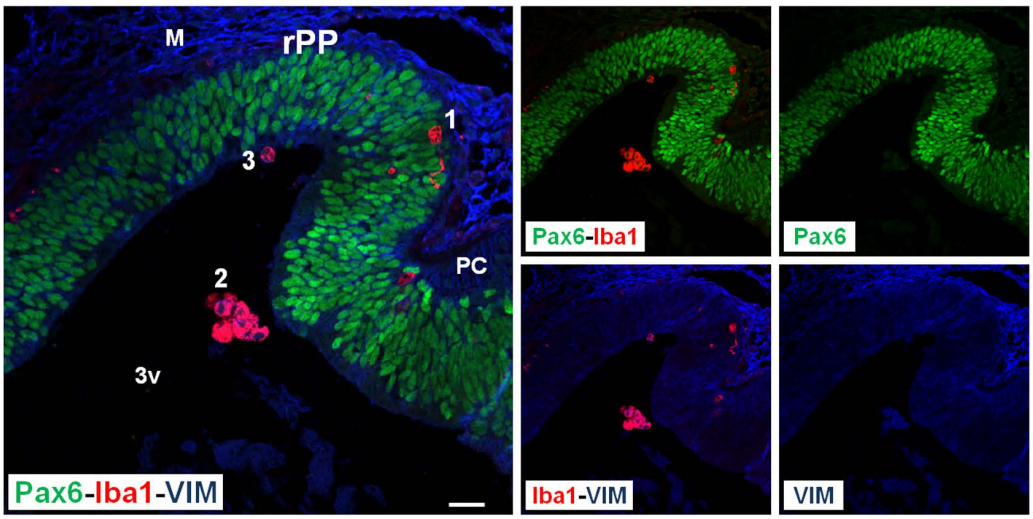
environments, under both physiological and pathological conditions (Grabert *et al.*, 2016; Matcovitch-Natan *et al.*, 2016; E Hirbec *et al.*, 2017).

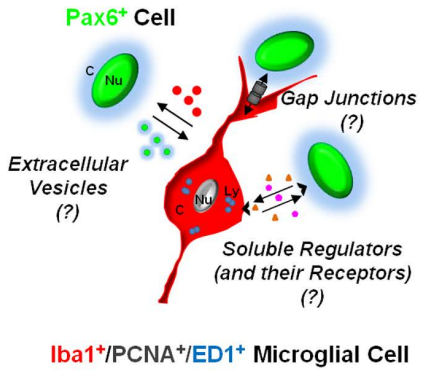
A



B

	PINEAL ONTOGENY		
STAGES	Primordium	Adult Gland	Senescent Gland
Density of Pax6⁺ Cells	High (with apparent homogeneous phenotype)	Low (with diverse phenotypes)	Unknown
Density of Iba1⁺/PCNA⁺/ED1⁺ Microglial Cells	Relatively uniform (with a slight but significant increase in the mature gland)		Unknown
Microglia-Pax6⁺ Cell Interactions (contact and/or engulfment)	Low	High	Unknown
Impact of Microglia on Pax6⁺ Cells	None or Promotion (?)	Suppression (?)	Unknown

C



D

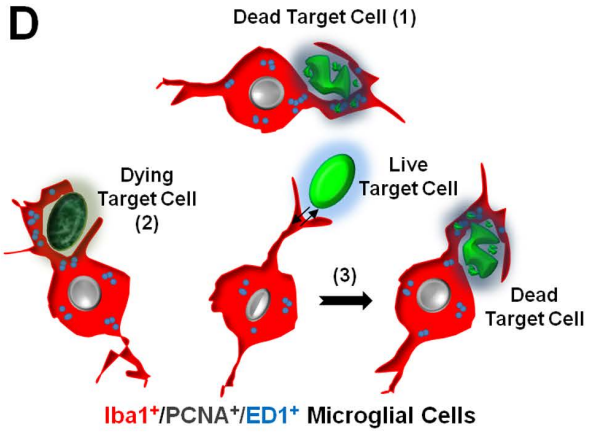


FIGURE 1: Microglia dynamics in the pineal gland. (A) In rat, Iba1$^+$ microglia colonize the Pax6$^+$/VIM$^+$ pineal primordium (rPP) from its very early formation (E15: Embryonic day 15). Microglia colonization stems from meninges (M; 1) and also from the subjacent third ventricle (3v; 2 and 3). Iba1: Ionized calcium-binding adaptor protein 1, macrophage marker (red). Pax6: Paired box 6, essential ontogenetic transcription factor, marker of precursor cells (green). PC: Posterior commissure. VIM: Vimentin, intermediate filament protein. Confocal microscopy, 60X, scale bar: 20 µm. (B) Dynamics of Iba1$^+$/PCNA$^+$/ED1$^+$microglia, Pax6$^+$ cells and their interactions throughout pineal ontogeny (Ibañez Rodriguez et al., 2016). ED1: also known as CD68: Cluster of differentiation 68, lysosomal marker. PCNA: Proliferating cell nuclear antigen. (C) Potential mechanisms of microglia-Pax6$^+$ cell interactions in the pineal gland. C: Cytoplasm. Ly: Lysosome. Nu: Nucleus. (D) Potential mechanisms of cell disposal by microglial cells. Microglia contact and engulf dead cells and cell debris (1) or dying cells (2). Microglial cells could also induce the death of stressed-but-viable cells (3).

The early microglia colonization of the CNS has led researchers to speculate about their roles in the fundamental developmental processes of neurogenesis, gliogenesis, vasculogenesis and angiogenesis. Microglia constantly interrogate and respond to cells in their environments, which could serve to communicate developmental tasks to microglia (Reemst *et al.*, 2016; Posfai *et al.*, 2018). Therefore, we can formulate the following questions: Do microglia have any preference for a particular cell type or cell stage or cell status? Is there any temporal window for microglia-cell interactions in a given CNS area or condition? Which mechanisms are involved in these cell-cell interactions? What is the impact on normal CNS ontogeny and maturation? How much influence do microglia-cell communications have in the onset and progression of neuropathologies? Even though microglia research has expanded greatly, answers to these questions are not yet clearly understood, and further investigation is needed.

As an example, our group recently characterized the pattern of microglia colonization and establishment in the pineal gland (Ibañez Rodriguez *et al.*, 2016). The pineal gland is a circumventricular organ that develops from the neural tube, like the rest of the CNS (Castro *et al.*, 2015). In mammals, the pineal gland is part of a complex circadian timing system (CTS) that influences the whole physiology of an organism. The pineal gland produces the nocturnal melatonin, a time-giver hormone that acts as both a CTS downstream effector to the rest of the body and as a CTS feedback regulator (Maronde and Stehle, 2007). In the rat embryo, we found that microglial cells disrupt the high homogeneity of the pineal primordium from its very early stages (Fig. 1A). Pineal precursor cells resulted immunoreactive for the ontogenetic transcription factor Pax6 (Paired box 6) and the intermediate filament protein vimentin (VIM). We also observed that microglia colonization stems from meninges and also from the subjacent ventricular cavity.

The close proximity between colonizing microglia and Pax6$^+$ precursors let us study the fate of the Pax6$^+$ cells and their potential regulation by the microglia throughout the entire pineal ontogeny. We showed that Pax6$^+$ cells give rise to the main pineal cell type, the pinealocytes, and also to a diverse glial population. Interestingly, the pineal microglia exhibited a continuously activated profile with an expanded cell body from which there were a few thick projections. In addition, pineal microglia resulted positive for Iba1 (ionized calcium-binding adaptor protein 1, macrophage marker), PCNA (proliferating cell nuclear antigen) and ED1 (also known as CD68: Cluster of differentiation 68, lysosomal marker). While the Pax6$^+$ cell population decreased exponentially during pineal development, the percentage of Pax6$^+$ cells contacted or engulfed by a relatively stable pool of microglial cells increased substantially (Fig. 1B). In the pineal gland of young adult rats, we observed that microglia-Pax6$^+$ cell interactions were modified by the local milieu. Surgical or pharmacological insults impacted differentially on the regulation of the Pax6$^+$ cell population by microglia, even though clustered microgliosis was commonly observed (Ibañez Rodriguez *et al.*, 2018). The impact of microglia-Pax6$^+$ cell interactions in the senescent pineal gland is still unknown (Fig. 1B). Together, these data support the concept that activated microglia are plastic and dynamic, and that microglia engage in a tightly regulated duplex dialog with Pax6$^+$ cells, which ultimately determines their fates. Further, we speculate that pineal microglia may either nurture or dispose Pax6$^+$ cells according to the stage of development or to the local milieu. The underlying mechanisms for this have not been characterized (Figs. 1B, 1C and 1D).

The dual actions of microglia, as suggested above, have been reported in relation to the main developmental processes that shape the CNS. In the developing cerebral cortex of different species, for example, microglia were proposed to either promote or suppress neurogenesis by upregulating or downregulating neural precursor cells, respectively (Cunningham *et al.*, 2013; Arno *et al.*, 2014; Barger *et al.*, 2018). The precise mechanisms responsible for the opposing actions of microglia are not yet well defined and further research is needed. Microglia may interact with non-microglial cells in a bidirectional manner, through some combination of released soluble signaling factors, extracellular vesicles, and gap junctional communication (Fig. 1C) (Matarredona *et al.*, 2018). Typical soluble signaling can be executed by cytokines, growth factors, neurotransmitter and hormones. Apoptotic bodies, microvesicles and exosomes containing lipids, proteins and miRNAs, might be also a source of microglia-precursor cell signaling. It has been suggested that connexin-based pores on the microglia membrane might be active gap junctions, although there are some discrepancies about this. If so, this might be a third avenue for cell-cell communication. Many of the effectors and regulators of the microglia in the CNS were identified in the pineal gland by global transcriptome analyses (Bailey *et al.*, 2009; Hartley *et al.*, 2015), but these same factors in the pineal gland have not been fully verified. A duplex microglia-pinealocyte network was proposed, which modulates melatonin synthesis under inflammatory conditions via the toll-like receptor 4 (TLR4), the tumor necrosis factor (TNF) and its receptor (TNFR1), and the nuclear factor kappa B (NF-kB) pathway (da Silveira Cruz-Machado *et al.*, 2012). The contribution of Pax6$^+$ cells to this modulatory network is unknown.

Microglia-cell interactions may or may not terminate with the internalization and subsequent degradation of the target cell (Fig. 1D). During CNS development, microglia perform phagocytosis to remove the excess of cells that are produced or made redundant during the basic neurogenic and gliogenic processes. In the mature CNS, microglia also eliminate cells during adult neurogenesis, and as needed for senescence, and for damage repair or remodeling (Brown and Neher, 2014). It was also shown that stressed-but-viable cells can be contacted and engulfed by microglia in a process known as phagoptosis (Fig. 1D) (Brown and Neher, 2014). More recently, autophagy has been related to microglia in CNS senescence and disease (Plaza-Zabala *et al.*, 2017). Our studies in the rat pineal gland and also cerebral cortex research by other investigators have revealed that precursor-like cells without signs of death or classic apoptosis were approached and internalized by microglial cells (Cunningham *et al.*, 2013; Ibañez Rodriguez *et al.*, 2016; Ibañez Rodriguez *et al.*, 2018). Mechanisms for cell disposal by microglia are not well understood, especially in the pineal gland, and further

research is needed under both normal and insult-responsive conditions.

Together, these data suggest that a (dys)-regulation of the bidirectional communication between microglia and precursor cells might affect CNS formation and maturation in ways that might impact its ability to function and maintain homeostasis. Precisely, miscommunication between microglia and other cells in the developing and mature CNS resulting in altered phagocytosis, has been pointed out as a leading mechanistic element in congenital neurological defects, neuroinflammation, ischemia/stroke and epilepsy, and neurodegenerative disorders like Alzheimer's, Huntington's and Parkinson's diseases (Brown and Neher, 2014; Plaza-Zabala *et al.*, 2017; Cloarec *et al.*, 2018).

In conclusion, microglia are more than just the immunological warriors of the CNS. They modulate many processes that take place in the developing and mature CNS. Microglial cells continuously interrogate and respond to their milieu, sometimes via bidirectional interactions with surrounding cells. The pineal gland derives from the neural tube, as does the rest of the CNS. Like the CNS, the pineal gland is also colonized by microglial cells. Pineal microglial cells are constantly activated phagocytes with a preference for $Pax6^{+}$ cells. Recent work from our group proposed the pineal gland as a model to study phagocyte turnover and microglia regulation of precursor-like cells in health and in disease. Further research is needed to finely characterize the underlying molecular mechanisms, which may or may not share molecules and sequentiality of events with those that occur in the rest of the CNS.

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