Chapter 6

Summary, general discussion and future perspectives
6.1 Summary

Inflammation is a dynamic and complex process in which multiple cell types and signaling pathways are involved, meant to restore homeostasis after injury or pathogen invasion. Comparable with the role of macrophages in peripheral inflammation, microglia cells play a prominent role in neuroinflammation. On the one hand, the role of microglia is beneficial by maintaining homeostasis, and being involved in brain development, neurogenesis and ageing [1,2]. On the other hand, activation of microglia has been implicated in the pathogenesis of several neurodegenerative and neurological diseases, e.g. Alzheimer’s disease (AD), Parkinson’s disease (PD), multiple sclerosis (MS), stroke, and possibly schizophrenia and depression [2,3]. In healthy conditions, microglia appear as ramified cells, scavenging the brain. In pathological conditions, microglia adopt a more amoeboid shape and move to the site of inflammation. Activation phenotypes of microglia appear as a continuum, with on one end a neuroprotective, anti-inflammatory phenotype and on the other end a neurotoxic, pro-inflammatory phenotype. Activation of microglia is dynamic, and suggested to change dependent on the stage of a disease, or even the disease itself. Upon microglial activation, receptor expression (e.g. of purinergic receptors) is altered, and differs between activation phenotypes [2].

Purinergic P2X$_7$ and P2Y$_{12}$ receptors are considered to be involved in the neuroinflammatory response. Their level of expression on activated microglia is dependent on the state of activation. While P2X$_7$ receptor upregulation is associated with the pro-inflammatory phenotype, P2Y$_{12}$ receptor upregulation is associated with the anti-inflammatory phenotype. As neuroinflammation is a dynamic process, being able to study the expression of both receptors in vivo, could provide new insights in the exact role of microglial activation, in particular in the progression of neurodegenerative diseases. An excellent tool that can be used to serve this purpose is positron emission tomography (PET).

In chapter 1, a general introduction on the basic concepts of PET imaging and neuroinflammation is provided, in order to delineate the rationale behind the research described in this thesis.

Chapter 2 gives an overview of the most recent developments in the field of PET imaging of neuroinflammation. The main part of this overview is dedicated to PET tracers targeting the translocator protein 18 kDa (TSPO), which to date still is the most investigated target in imaging of microglial activation, both in preclinical and clinical studies. However, targeting TSPO comes with certain limitations, especially with respect to large differences in binding affinity of most tracers between human subjects, caused by the rs6971 single nucleotide polymorphism. Whereas focal and acute neuroinflammation in stroke and MS can be visualised fairly well using TSPO PET, imaging of neuroinflammation in AD is still hampered by low signal-to-noise ratios of the currently used tracers. Therefore, next to TSPO tracer development, also other existing and emerging molecular targets, including the P2X$_7$ receptor, for development of tracers for imaging of neuroinflammation are discussed in chapter 2.

Over recent years, the P2X$_7$ receptor gained increasing interest as a novel microglial target for in vivo imaging of neuroinflammation. In chapter 3, the synthesis of one of the first PET tracers
targeting the P2X7 receptor is described. Desmethyl precursor (8) was synthesised via a 7-step synthesis route, and subsequently reacted with [11C]methyl iodide to obtain [11C]A-740003 (scheme 1). Evaluation of this tracer in healthy rats revealed a moderate metabolic stability, but little to no brain uptake. Therefore, further in vivo evaluation of [11C]A-740003 was discontinued. Nonetheless, [11C]A-740003, as well as its tritium-labelled analogue [3H]A-740003, could be used in vitro to evaluate expression of the P2X7 receptor in animal models of neuroinflammation, which is described in chapter 4. Neuroinflammation can be induced locally in vivo in e.g. mice and rats by stereotactic injection of neurotoxins. Three different neurotoxins were used. Stereotactic injection of quinolic acid (QA) in striatum evokes a neuroinflammatory response, in which activated microglia predominantly adopt a pro-inflammatory phenotype. Autoradiography experiments on brain sections of this particular model revealed increased binding of [3H]A-740003 in the lesioned striatum compared with the contralateral striatum. Binding of [3H]A-740003 was shown to correlate with binding of [3H]PK-11195, a radioligand targeting TSPO, indicating a similar origin for upregulation of the P2X7 receptor and TSPO. Nevertheless, in another pro-inflammatory animal model of neuroinflammation, induced by intrastriatal injection of lipopolysaccharide (LPS), no difference in binding was observed between injected and non-injected striatum. Following intracerebroventricular injection of interleukin-4 (IL4), inducing an anti-inflammatory phenotype of activated microglia, no difference in binding of [11C]A-740003 was observed between affected and vehicle injected mouse brains. Taken together, in vitro autoradiography results in animal models of neuroinflammation are in line with the notion that P2X7 receptor upregulation is associated with the pro-inflammatory microglial phenotype rather than the anti-inflammatory phenotype.

Adamantanylbenzamide 1, a potent P2X7 receptor antagonist developed by AstraZeneca [4], was shown enter the CNS, but also showed a poor pharmacokinetic profile. Based on this literature lead, Wilkinson et al. [5] have designed a series in which the hydrogen atoms at the bridgeheads of the adamantane moiety were substituted by fluorine atoms, to prevent in vivo hydroxylation of this position. Indeed, increased stability was achieved in rat and mouse liver microsomes [5]. In chapter 4, carbon-11 methylation of the adamantanylbenzamide series (figure 1) and subsequent preclinical evaluation is described. [11C]1-4 could all be obtained in high radiochemical yield and with high radiochemical purity and molar activity, and thus all four tracers were evaluated in healthy rats. Organ distribution, including sufficient brain uptake, was similar for all tracers. Metabolite analysis confirmed significantly increased metabolic stability of [11C]4 (42 ± 2% of intact tracer 45 min p.i.) compared with [11C]1-3 (25 ± 1%, 15 ± 2% and 16 ± 1% of intact tracer 45 min p.i., respectively) in rat plasma. Based on these results, PET imaging was performed...
using $[^{11}C]4$ in a rat model with local overexpression of the human P2X$_7$ receptor, which was achieved via injection of an adeno-associated viral vector expressing the human P2X$_7$ receptor [6]. By unilateral injection of the human P2X$_7$ receptor expressing vector in striatum, the contralateral striatum could serve as internal control. Uptake of $[^{11}C]4$ was 1.5-fold higher in the human P2X$_7$ receptor expressing striatum compared with the contralateral striatum (SUVs 2.85 ± 0.69 vs. 2.30 ± 0.36) from 2 min p.i. throughout the remainder of the scanning time (60 min). Uptake of $[^{11}C]4$ could be blocked by pre-treatment with a non-structurally related P2X$_7$ receptor antagonist (JNJ-47965567; 30 mg·kg$^{-1}$, administered subcutaneously 45 min prior to tracer injection), indicating specific binding of $[^{11}C]4$ to the human P2X$_7$ receptor in vivo.

Moving towards a more clinically relevant use of $[^{11}C]4$, in vitro autoradiography was also performed on sections of post mortem brain material of human AD patients. Although immunohistochemical staining showed a slight increase of P2X$_7$ receptor expression in AD patients when compared with post mortem brain material of non-neurological age-matched controls, no significant difference in binding of $[^{11}C]4$ was observed. This may be a matter of resolution, however, as already shown previously with TSPO PET, AD may not be the optimal disease for validation of novel tracers for neuroinflammation, as the response in AD is mild and non-focal, compared with a more acute and focal microglial response in for instance MS or stroke.

To identify novel targets for PET imaging of neuroinflammation, co-expression networks were generated from publicly available gene expression datasets, which is described in chapter 5. Analysis of expression data was guided by pre-defined criteria: i) expression preferentially in microglia compared with macrophages; ii) higher expression in anti-inflammatory microglia phenotype compared with non-stimulated or pro-inflammatory phenotype; iii) similar modulation in rodents as in humans. Apart from biological criteria, selected genes were also classified from a radiopharmaceutical perspective, e.g. preference for cell surface expression over intracellular expression and the availability of selective ligands in the literature to be used as lead compounds for PET tracer development. Keeping all criteria in mind, the P2Y$_{12}$ receptor came up as a promising translational biomarker. Subsequently, in in vitro experiments, P2ry12 mRNA was shown to be increased after treatment of primary human microglia cultures with IL4, but not after treatment with LPS or vehicle. In addition, a similar experiment in primary human macrophage cultures revealed no mRNA expression of Pry12 in any condition. Taken together, these data confirmed the potential of the P2Y$_{12}$ receptor as a target for PET imaging of the anti-inflammatory phenotype of microglia. As the P2Y$_{12}$ receptor is a well-known drug target for anti-coagulants, numerous selective antagonists with high affinity for this receptor have been developed. A highly potent P2Y$_{12}$ receptor antagonist [7] was selected as lead for
development of the first PET tracer targeting the P2Y$_{12}$ receptor, [${}^{11}$C]5, which was obtained from precursors 3 and 6 via rhodium-mediated carbon-11 carbonylation (scheme 2). In vitro evaluation of [${}^{11}$C]5 indeed revealed higher tracer binding in IL4 injected mouse brains compared with vehicle injected mouse brains. Surprisingly, autoradiography on brain sections of a mouse model of stroke (MCAO) showed decreased tracer binding in the affected hemisphere compared with the contralateral hemisphere, and these results were confirmed by immunohistochemical staining for the P2Y$_{12}$ receptor. Decreased binding was most pronounced 3 days after MCAO, suggesting microglia switch to a more anti-inflammatory phenotype at later time points after stroke (7 and 10 days). In addition, the same phenomenon was observed in post-mortem brain material of a patient who died from a stroke. These results confirm down-regulation of the P2Y$_{12}$ receptor in the pro-inflammatory microglial phenotype, and would allow for in vivo imaging of the dynamics of activated microglia in disease. However, in vivo evaluation in rats proved that [${}^{11}$C]5 is rapidly metabolised, likely due to cleavage of the ester moiety. Furthermore, no uptake of the tracer was observed in brain, and combined with the high uptake in duodenum, this points towards [${}^{11}$C]5 being a substrate for P-glycoprotein (P-gp), a brain efflux transporter.

![Scheme 2: Radiosynthesis of P2Y$_{12}$R antagonist [${}^{11}$C]5, described in chapter 5.](image)

### 6.2 General discussion and future perspectives

In this thesis, overexpression of the P2X$_7$ receptor was shown in an animal model of QA induced pro-inflammatory microglial activation. Although in an LPS induced animal model of pro-inflammatory microglial activation P2X$_7$ receptor overexpression could not be shown, others have been able to show upregulation of the P2X$_7$ receptor in brain after systemic administration of LPS [8,9]. The differences in results may be explained by the high variability in results observed following LPS induced neuroinflammation, due to experimental differences (e.g. source of LPS) [10]. Taken together, these results suggest that P2X$_7$R may indeed be a promising new target for PET imaging of the pro-inflammatory microglial phenotype in vivo. The P2X$_7$ receptor antagonist [${}^{11}$C]4 was shown to specifically bind to the human P2X$_7$ receptor in vitro and in vivo in a rat model using vector-mediated overexpression of the receptor.

Unfortunately, in post-mortem brain tissue of AD patients, no difference in tracer binding could be observed compared with healthy brain tissue. However, similar to clinical imaging studies in AD patients with tracers targeting TSPO, AD might not be the optimal disease to study and validate novel tracers of neuroinflammation. In contrast, using TSPO PET in MS, differences between patients and healthy volunteers can be observed fairly well [11,12], possibly due to the more focal and acute nature of the neuroinflammatory response. In line with this, recent autoradiography data in post-mortem human brain sections of MS patients revealed that P2X$_7$ receptor expression is increased in active lesions in MS [13]. In addition, the same phenomenon
was observed in a rat model of MS, in which the P2X7 receptor expression was increased at the peak of the disease [13]. These results correspond to a recent study with $[^{11}C]GSK1482160$, another newly developed tracer targeting the P2X7 receptor, in which increased tracer binding in sections of lumbar spinal cord of a rat model of MS was observed in vitro [14]. However, this marked increase in tracer uptake could not be observed in vivo, as the affinity of $[^{11}C]GSK1482160$ is 100-fold lower for rodent receptors compared with the human receptor [14]. Although to a lesser extent, a similar problem arises for $[^{11}C]4$. Therefore, $[^{11}C]4$, or as it is called now, $[^{11}C]SMW139$, warrants clinical rather than further preclinical evaluation in vivo. To this end, next to the validation of a GMP compliant synthesis, SMW139 was shown to be non-toxic in an extended single microdose toxicity study (0.12 mg·kg$^{-1}$) in rats, and proof-of-concept clinical studies using $[^{11}C]SMW139$ in MS and PD patients are currently ongoing.

Next to development of a tracer targeting the P2X7 receptor that is potentially selective for the pro-inflammatory phenotype of activated microglia, it would be of additional value to identify a novel target for PET imaging specific for the anti-inflammatory phenotype. This would allow for studying the dynamic process of microglial activation in both health and disease in vivo. In addition, by being able to visualise both ends of the neuroinflammatory spectrum in vivo, treatment effects (e.g. of anti-inflammatory agents) could be studied. Therefore, in chapter 5 of this thesis, the feasibility of a rationale-based approach for the identification of novel targets for PET imaging of the anti-inflammatory phenotype of activated microglia was demonstrated. Such an approach, from gene identification to PET tracer development, is widely applicable to all imaginable targets.

Although in the end, the carbon-11 labelled tracer developed for targeting the P2Y$_{12}$ receptor could not be used in in vivo studies due to the fact that it does not enter brain, increased tracer binding was observed in vitro in IL4 induced neuroinflammation. Therefore, the P2Y$_{12}$ receptor is a feasible target for imaging of the anti-inflammatory microglial phenotype. In addition, decreased tracer binding and immunohistochemical staining for the P2Y$_{12}$ receptor was observed in vitro in a stroke model of mouse, as well as in brain sections of a human stroke patients. Recently, in an immunohistochemical analysis of post-mortem brain tissue of MS patients, a loss of P2Y$_{12}$ receptor expression was found in active lesions, but also in unaffected white matter [15]. Corresponding to these results, our group has shown a decrease in tracer binding in autoradiography experiments both in human MS and in a rat model of MS [13]. Together these results indicate that the P2Y$_{12}$ receptor is an excellent, translational target to study microglial activation in disease progression, and warrant further validation of the receptor in vivo. However, this requires a PET tracer that enters the brain, and due to the fact that the P2Y$_{12}$ receptor is a well-known drug target for treatment of blood clotting and thrombosis, most selective compounds are far too polar to cross the blood-brain barrier. As indicated in chapter 1, in addition to high affinity and selectivity for the target, a good PET tracer for brain imaging requires additional properties. For instance, a low molecular weight (<500 Da) and a topological polar surface area (tPSA) below 90 Å$^2$, have been suggested to increase the chance of compounds to cross the blood-brain barrier by passive transport. Another major obstacle
in CNS drug development is the presence of brain efflux transporters, e.g. P-gp, for which lipophilic compounds of low molecular weight tend to be substrates [16]. A medicinal chemistry approach, specifically focused on finding an optimum between features as tPSA, lipophilicity and, possibly most difficult, chances of being P-gp substrate [17], could allow for identification of a PET tracer able to target the P2Y$_{12}$ receptor in the living brain.

To conclude, the development of a promising PET tracer targeting the P2X$_7$ receptor for imaging of the pro-inflammatory phenotype of microglia, together with the validation of the P2Y$_{12}$ receptor as a promising target for imaging of the anti-inflammatory phenotype, has brought us one step closer to having the tools in hand to image the dynamics of microglia in the living brain. When combined, P2X$_7$ and P2Y$_{12}$ receptor imaging may provide insights in the dynamic continuum of pro- and anti-inflammatory microglial activation in disease onset and progression. In addition, modulation of microglia could be monitored in vivo, which could allow for identification of new treatment opportunities in neurodegenerative diseases.

References


