Chapter 1

General introduction
1.1 Neuroinflammation

Inflammation is a dynamic and complex process that is present in all vertebrates, and which has the ultimate goal to restore homeostasis after an insult, such as injury or infection. This process involves multiple cell types and signalling pathways [1]. Although inflammation is intended as a healing process, persistent inflammation can also become detrimental. An inflammatory response can also occur in the central nervous system (CNS), hence termed neuroinflammation. Although the CNS is separated from the periphery by the blood-brain barrier, and distinct cells are involved in neuroinflammation compared with systemic inflammation, communication exists between the peripheral immune system and the CNS [1,2]. Comparable with the role of macrophages in peripheral inflammation, microglia cells are often described as the first line of defence in the CNS.

Microglia account for around 10% of brain cells, which make them the most abundant immune cells in the CNS. Activation of microglia has been implicated in the pathogenesis of several neurodegenerative and neurological diseases, e.g. Alzheimer’s disease (AD), Parkinson’s disease, multiple sclerosis, stroke, and possibly schizophrenia and depression [2,3]. Apart from their suggested role in disease, microglia also play an important role in brain development, neurogenesis and ageing [1,3]. Ramified microglia constantly survey the brain to detect anything out of the ordinary, by contacting neurons, astrocytes and monitoring the functional state of synapses [3]. Upon activation, microglia change shape and move to the site of inflammation, dependent on whether their function is more associated with neurotoxicity (pro-inflammatory) or neuroprotection (anti-inflammatory). Microglial activation is a highly dynamic process, and is nowadays seen as a continuum of activation phenotypes, with on one end the neurotoxic, pro-inflammatory phenotype and on the other end the neuroprotective, anti-inflammatory phenotype [3,4]. The activation status is dependent on different stimuli, and most likely, microglia can also switch between phenotypes during disease progression. Furthermore, the exact role of both pro- and anti-inflammatory microglia in neurodegeneration is still not fully understood, and may also differ depending on the disease, or even the stage of the disease [3]. Along with their morphological changes, receptor expression (e.g. of purinergic receptors) is altered, and differs between activation phenotypes. Such a change in receptor expression can be visualised with molecular imaging techniques, for instance positron emission tomography (PET).

1.2 Positron emission tomography

PET is a highly sensitive, non-invasive molecular imaging technique. As opposed to imaging techniques such as X-ray, computed tomography (CT) and magnetic resonance imaging (MRI), which provide structural, anatomical and functional information, nuclear imaging techniques such as PET and single-photon emission computed tomography (SPECT) can provide functional and molecular information about biological processes in living patients [5]. PET has the advantage over SPECT of a higher sensitivity, and, in addition, the PET signal allows for quantification.
Nuclear imaging techniques rely on radioactive decay of unstable nuclei, due to an imbalance in the number of protons and neutrons. Neutron deficiency of nuclei can be resolved by either electron capture or emission of a positron. Whereas SPECT is based on detection of gamma emission, for instance resulting from electron capture, for positron emission tomography (PET), as the name already suggests, emission of a positron is required. An emitted positron will travel a short distance in tissue, on its way losing kinetic energy through collisions with electrons, until annihilation with an electron occurs. Upon annihilation, two 511 keV photons are emitted traveling in opposite direction of each other. Scintillation detectors, usually positioned in a circle, can detect both photons as a coincidence event (i.e. simultaneously), thereby allowing for localisation of the line along which the annihilation took place (Figure 1).

Thus, PET requires the use of a radiopharmaceutical labelled with a positron emitting radionuclide (Table 1), which can be produced in a cyclotron and subsequently chemically incorporated in a target molecule [6]. A variety of biochemical processes in the living body can be visualised, as radioactively labelled molecules can be designed to target specific enzymes or receptors. Substitution of a stable nuclide (e.g. $^{12}\text{C}$ or $^{19}\text{F}$) with the corresponding radioactive isotope does not alter the chemical structure of the target compound, and thus the tracer displays identical physicochemical and biological properties compared with the non-labelled compound. Therefore, next to imaging of biochemical processes in vivo, PET is increasingly used as a tool in drug development. As the PET signal is purely based on the distribution of radioactivity, which reflects a biological process, it mostly does not provide anatomical information. Therefore, PET is often combined with CT or MRI to allow for anatomical localisation of the measured signals.
The exact role and reactivity of microglia in the neuroinflammatory response in vivo is still not fully understood. PET is an excellent tool to study the behaviour of microglia in the living brain, depending on the availability of the right radiopharmaceuticals. In general, the preferred biological target for a radiopharmaceutical is membrane-bound, with the binding site located at the extracellular site. Ideally, target expression should be upregulated, or at least altered, under pathological conditions compared with the healthy situation. Next to general radiopharmaceutical characteristics such as nanomolar affinity and high selectivity for its target, radiopharmaceuticals for brain imaging require some additional properties [7]. As the brain is separated from the blood by the blood-brain barrier, a useful radiopharmaceutical for brain imaging should cross this barrier by passive transmembrane diffusion, unless imaging of active transport via brain efflux transporters (e.g. P-glycoprotein) is desired [8]. In order for passive diffusion to occur, the ideal tracer should have low molecular weight (<500 Da) and a polar surface area below 90 Å². In addition, the capacity to form hydrogen bounds should be limited [9], and lipophilicity should be optimal (cLogD7.4 1-5) [10]. This is two-fold, as too hydrophilic compounds will not be absorbed by the blood-brain barrier, whereas too lipophilic compounds will be absorbed, but fail to enter the aqueous environment of the brain’s interstitial fluid [10]. All of these properties need to be balanced to obtain ‘the ideal’ radiopharmaceutical for brain imaging, which underlines the challenges in the field of brain PET imaging.

1.3 PET imaging of neuroinflammation

1.3.1 Current state of the art

A thoroughly investigated target in PET imaging of neuroinflammation, is the translocator protein 18 kDa (TSPO). TSPO is located on the outer mitochondrial membrane, and next to its expression in several peripheral organs, it is also found in the brain. Expression of TSPO on microglia is minimal in the healthy brain, but is highly upregulated during microglial activation [11]. To date, over 80 radiotracers targeting TSPO have been reported, of which \((R)-[\text{11C}]\text{PK11195}\), developed in the 1980’s, is still most used [12-14]. Although PET tracers targeting TSPO allowed for the characterisation of neuroinflammation in many preclinical and clinical studies, targeting TSPO has some limitations. \((R)-[\text{11C}]\text{PK11195}\) itself suffers from low brain uptake and high non-specific binding [14], which led to the development of the so-called second generation TSPO tracers. However, for several of these second generation tracers, highly variable tracer binding between subjects is observed, caused by a genetic polymorphism \((rs6971)\) in the human gene encoding TSPO [15], which complicates quantification. Furthermore,
expression of TSPO is not restricted to activated microglia only, but also appears on astrocytes and macrophages, which can infiltrate the brain in case of a compromised blood-brain barrier. Therefore, PET tracers targeting TSPO do not show microglial activation exclusively, but a more general inflammatory process [11]. In addition, expression of TSPO is upregulated in both the pro- and anti-inflammatory phenotype of microglia [11,16], and using TSPO as a target for molecular imaging can therefore not provide insight on the exact role of either phenotype. As receptor expression differs between different phenotypes of activated microglia, targeting receptors of which the expression is only upregulated in a specific phenotype, could allow for monitoring of disease progression and treatment effects.

1.3.2 Novel approach: targeting purinergic receptors

Purinergic receptors can broadly be classified in two types; P2X and P2Y receptors. P2X receptors are ligand-gated cation channels. Upon binding of adenosine triphosphate (ATP), the channel is permeable to Na⁺, K⁺ and Ca²⁺ [17]. At present, seven subtypes of the P2X receptors are identified, most of which are linked to neuroinflammation. The strongest body of evidence for involvement in mediation of the neuroinflammatory response exists for the P2X, receptor subtype [17-20]. In the CNS, P2X, receptors are expressed mainly on microglia, but expression has also been found on astrocytes, oligodendrocytes and neurons. Affinity of ATP for the P2X, receptor is low, and therefore millimolar concentrations of extracellular ATP are required for P2X, receptor pore formation, which in turn enables the cell to excrete molecules up to 900 Da, e.g. cytokines and chemokines. ATP activation of the P2X, receptor leads to initiation of the neuroinflammatory cascade through activation of the NLRP3 inflammasome, accompanied by increased release of pro-inflammatory cytokine IL-1β. Therefore, activation of P2X, receptors is associated with the pro-inflammatory phenotype of microglia.

P2Y receptors are G-protein coupled, metabotropic receptors, and so far eight subtypes have been identified, all of which are expressed in the CNS [17]. The P2Y₁₂ receptor is a well-known antithrombotic drug target, as activation of the P2Y₁₂ receptor by ADP leads to platelet aggregation, whilst inhibition prevents this process. In the CNS, P2Y12 receptors are expressed on microglia [21], in which P2Y₁₂ receptor expression is involved in process extension and migration to sites with increased ATP concentration, often associated with brain injury. In addition, it has been shown that the P2Y₁₂ receptor is downregulated in the pro-inflammatory microglial phenotype [22], but upregulated in the more protective anti-inflammatory phenotype [21]. Another advantage of the P2Y₁₂ receptor is that it is not expressed on monocytes and macrophages, and therefore it is possible to discriminate between infiltrating monocytes and microglia.

Evidently, both P2X, and P2Y₁₂ receptors are promising targets for molecular imaging of neuroinflammation or, more specifically, for imaging of the pro- and anti-inflammatory phenotypes of activated microglia, respectively.
1.4 Aims and thesis outline

The aim of the research described in this thesis was to develop radiotracers that are able to visualise neuroinflammatory responses in the brain in vivo, particularly targeting the purinergic P2X$_7$ and P2Y$_{12}$ receptors in an attempt to selectively detect pro- and anti-inflammatory microglia. The field of neuroinflammation imaging is of high interest, and scientific literature on this topic is expanding fast, in particular related to clinical imaging targeting TSPO. Chapter 2 describes recent developments in this field, summarizing both TSPO and emerging targets for imaging of neuroinflammation. Chapter 3 describes the synthesis and preclinical evaluation of the selective P2X$_7$ receptor antagonist \([^{11}C]A-740003\). Chapter 4 describes radiolabelling of four potent, brain penetrating P2X$_7$ receptor antagonists. Rats overexpressing the human P2X$_7$ receptor were subjected to PET scans using the most promising tracer, and in vitro evaluation of this tracer was performed on post-mortem brain tissue of AD patients as well as non-neurological controls. Chapter 5 describes the discovery and biological evaluation of a novel target for imaging of neuroinflammation, the P2Y$_{12}$ receptor. A potent P2Y$_{12}$ receptor antagonist was synthesised and radiolabelled via a rhodium-mediated carbon-11 carbonylation reaction. Subsequently, this novel tracer was evaluated in a mouse model of neurotoxin induced neuroinflammation, a mouse model of stroke and post-mortem brain tissue of a stroke patient.

References

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