The role of the P2X7 receptor as a key player in neuronal development and implications for schizophrenia animal models

PhD thesis

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1. Introduction

Extracellular ATP is an important signalling molecule that regulates several cellular functions and activates purinergic P2 receptors, which are expressed in the early embryonic stage during brain development, thus influencing cellular differentiation, proliferation, and apoptosis. At high levels, extracellular ATP operates as a "danger" molecule under pathologic conditions through purinergic receptors, including the ionotropic P2X7 receptor (P2X7R). The ionotropic receptors P2X and particularly the P2X7R have received considerable attention in the past decade because of their involvement in diseases of the central nervous system, including psychiatric disorders. Indeed, P2X7R endogenous activation is associated with neurodevelopmental disorders. However, its function during early embryonic stages remains largely unclear despite the fact that purinergic signalling has been shown to play a role in physiological brain development.

This PhD thesis aims to assess the regulatory effect of P2X7R on neuronal outgrowth and morphology in primary cultures of murine hippocampal neurons and acute hippocampal slices. As well this thesis will focus on the study of role of the receptor on the pathogenesis of schizophrenia. Schizophrenia is regarded as a neurodevelopmental psychiatric disorder, a dynamic disorder, in which changes in brain development and maturation might be because of the interaction of various genetic, epigenetic, and environmental factors.

1.1. P2X family with emphasis on the P2X7 receptor

The purinergic P2X receptors are trimeric ligand-operated channels. Each subunit comprises two transmembrane domains presenting an intracellular C and N-termini and a large ectodomain. Three ATP binding sites are formed between the interface of two subunits containing highly conserved residues. The C-terminus is formed by a cysteine-rich region, which serves as an anchor to the cell membrane, and a 200-residue extended region denominated as "cytoplasmic ballast". When P2X7R is persistently activated, it leads to apoptosis and cell death, consequently potentiating the P2X7R response by releasing more ATP into the extracellular space (1). This process entitles P2X7R as the gatekeeper of the immune responses in assembling the inflammasome (NLRP3/ASC/Procaspase-1). The cleavage and activation of Caspase-1 promote the

process of pro-IL-1β to IL-1β for further secretion in the extracellular space. Finally, the whole cascade results in the induction of proliferation, recruitment and activation of macrophages, microglia, lymphocytes and the other inflammatory cytokines such as TNF-α or Interleukin 6 (IL-6) (2). For these reasons, P2X7R has been associated with inflammatory pathological conditions in neuroinflammatory and neurodegenerative diseases contributing to disease progression.

1.2. P2X7 receptor in physiological conditions: new insights in brain development.

A wealth of data indicates now that P2X7R is also involved in the regulation of physiological functions such as neurotransmitter release, memory and cognition, as well as the development of the nervous system. However, it is still being determined how their presence and absence impact the development and morphology of the primary pyramidal neurons from the hippocampus *in vitro.*

It has been hypothesised that P2X7R promotes initial proliferation as its expression is higher in rat telencephalon neurospheres in earlier days *in vitro* while getting down-regulated during the 14 days in culture during neuronal differentiation (3). During development, P2X7R seems involved in necrosis and apoptosis (4), whereas other *in vitro* studies demonstrated different regulatory effects in axonal growth and branching (5). Therefore, P2X7R might be related to regulating the uncontrolled differentiation and migration of cells during neurogenesis. Finally, it is hypothesized whether the P2X7R role in maintaining both the neural stem and progenitor pools via inducing the proliferation or apoptosis might depend mainly on the microenvironment (6). Therefore, P2X7R might present different expression activity patterns to guide proliferation and differentiation processes into neuronal cells.

In adult neural progenitor cells, which are located in the neurogenic niches of the brain, P2X7R is still present (7). It has been recently reviewed that P2X7R plays at least three different roles in the adult hippocampus, influencing the hippocampal neurogenic niche, depending on the extracellular ATP concentration. At low extracellular ATP, P2X7R is believed to mediate calcium transduction signals regulating biological functions, such as proliferation and differentiation in adult neurogenic niches. Both in the presence of inflammation, then the high concentration of ATP, or, on the opposite

physiological conditions, at a total absence of the nucleotide, P2X7R might elicit different responses, such as promoting cell death or phagocytosis, respectively (8).

Overall, the expression and function of P2X receptors vary depending on the brain area and cells involved. Purinergic signalling is hypothesized to have multiple roles in neurogenesis and plastic remodelling during development, and its later downregulation may prevent the uncontrolled growth of progenitor cells. Consequently, purinergic receptor subtypes transiently expressed during brain development suggest that nucleotides and nucleosides might affect stage-specific developmental processes. This also highlights the importance of ATP in neuronal development and growth. Further research is necessary to fully understand the function of purinergic signaling and especially the P2X7 receptor, in neurogenesis and plastic remodelling during development.

1.3. P2X7R and schizophrenia: a key modulatory element in neuroinflammation

Schizophrenia is a chronic mental disorder up to an estimated prevalence of 0.5% to 1%, with a young-adulthood onset characterized by an array of psychotic symptoms, deprivation of emotional responses, and cognitive impairments associated with substantial comorbidities such as anxiety, substance abuse and depression, leading to a high incidence of suicide attempts, which is why is considered one of the leading cause of disability (9). The multiple symptoms range is grouped into positive, cognitive or negative symptoms.

Several epidemiological and genetic studies present the role of inflammation and immunity in schizophrenia associated with numerous infectious agents identified as risk factors and increased serum levels of pro-inflammatory cytokines. P2X7R as a significant driver of inflammation is a key mediator for ATP release in astrocytes and activated microglia. A result of high extracellular ATP and sustained receptor activation will lead to pore formation. Consequently, an efflux of intracellular components will, in turn, maintain elevated ATP levels in the extracellular space, which will continuously activate P2X7R and will eventually lead to cell death. Therefore, both the regulatory role of P2X7R in the inflammatory response and as a promoter of an immune response pose the receptor as a potential therapeutical target in psychiatric diseases, including schizophrenia. Whether inflammation during developmental stages influences the

development of SCZ as a neurodevelopmental disease, it was shown how purinergic modulation of subplate neuron activity might lead to disruptions in cortical development (10) and cognitive disturbances in neurodevelopmental disorders such as autism or schizophrenia upon a postnatal challenge.

Maternal immune activation (MIA) models involve injecting pregnant rodents with an immunogen to simulate a maternal immune-inflammatory response, leading to neurodevelopmental disruptions in the offspring, which can manifest as spatial and social deficits or anxiety-linked behaviours. The immunogens commonly used in MIA models include polyinosinic: polycytidylic acid (PIC), influenza virus, and lipopolysaccharide (LPS). Both PIC and LPS induce a robust innate reaction that precedes cytokine production, inflammation, and fever. Horváth and colleagues (11) demonstrated the role of P2X7R in neurodevelopmental disease for the first time in an MIA-induced autism-like behavioural model where endogenous receptor activation is needed to develop an autistic phenotype. Overall, human and rodent studies present shreds of evidence that P2X7R has a potential role as a mediator in early immune responses and the development, severity and, consequently, possible treatment responsiveness of schizophrenia. However, further research is necessary to fully understand the mechanism of action of the endogenous activation of the P2X7 receptor in the development of positive, negative and cognitive symptoms in an MIA model of schizophrenia.

2. Objectives

Two main objectives were established to study the role of P2X7R in the development of individual neurons and neurodevelopmental psychiatric disorders. Consequently, it was explored how its pathological activation is associated with schizophrenia-like behaviours in rodent animal models. Therefore, P2X7R was studied in detail in physiological and pathological conditions during development to young adulthood. The specific objectives were:

1. Establish the cell-specific expression and function of P2X7R in physiological conditions during development. Then:
    a. To investigate the role of P2X7R in dendritic outgrowth during neuronal development, proliferation, and maturation in the hippocampus.
    b. To assess the cognitive performance of P2X7R-deficient mice at different stages of development and youth to determine the role of the receptor on cognitive performance.
2. Establish the cell-specific expression and function of P2X7R in pathological conditions in schizophrenia mouse models.
    a. To better understand the over-activation of P2X7R in a mouse PCP model of schizophrenia.
    b. To determine the effects of endogenous activation of P2X7R on dendritic outgrowth *in vitro* and its behavioural outcome in a neurodevelopmental model of schizophrenia.

3. Methods

3.1 Experimental time-flow for experiments *in vitro*

To investigate the impact of P2X7R deficiency on neuronal growth, we bred P2rx7+/+ (WT) mice and P2rx7-/- mice (knockout; KO), respectively. Control and KO primary hippocampal neurons were maintained until day *in vitro* (DIV) 10 when the dendrites were fully developed. In order to retrain the study on the role of P2X7R on neuronal growth, the indirect effect of astroglia on neurons was blocked by adding cytosine-arabinofuranoside (CAR, 10μM), an anti-mitogen agent at DIV 3. To study P2X7R expression in our model, RNA was collected each day *in vitro* for ten days and the transcript level of the *P2rx7* gene was measured by RT-qPCR and validated with Western Blot. Consequently, to study neuronal activity, calcium imaging was performed in KO and WT primary hippocampal neurons to validate the maximum expression level in both RT-qPCR and Western Blot.

Then, transfecting cells with a plasmid encoding GFP under a synapsin promoter at DIV9 allowed the study of the morphology of individual neurons and dendrites in a given primary hippocampal neuron as it fulfils the whole neuron with green fluorescence. As a control for GFP transfection, we stained transfected primary neurons for MAP2, a neuron-specific cytoskeletal protein marker that specifically stains dendrites. Together, with the lower effect of the transfection compared to the whole immunostaining of MAP2, we can trace several isolated neurons to visualise their whole structure. Then, the morphology of these neurons was compared with that of primary neurons with either genetic deficiency or pharmacological blockade of P2X7R by Sholl analysis (Fig. 1).

Finally, when needed, high-performance liquid chromatography (HPLC) was used to quantify the release of adenine nucleotides (ATP, ADP, AMP) and adenosine (Ado) from the culture medium.

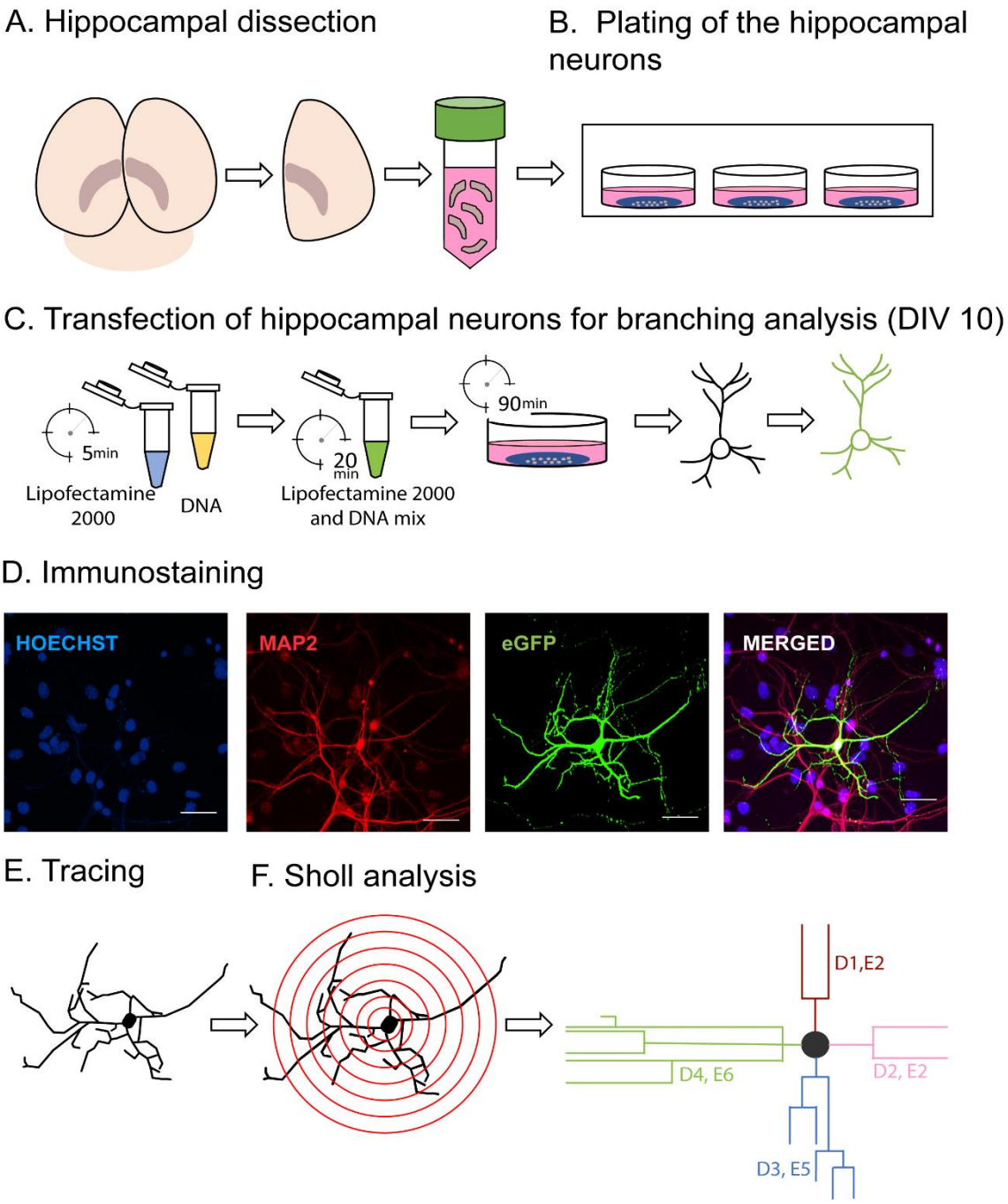

Figure 1. Scheme of the primary hippocampal neurons preparation and further morphological studies with Sholl analysis.

3.2. Maternal immune activation model: experimental design.

Maternal immune activation was used as a model for triggering schizophrenia-like behaviour in the offspring. Intraperitoneal poly(I:C) (PIC) injections during pregnancy

activate antiviral pattern recognition receptors, such as TLR3 and PKR, in the mother and thereby activate the maternal immune system.

Morphological correlates of the disease were studied in *vitro* primary cultures following the same outline previously described from the hippocampus dissected from control and PIC-treated embryos. For the *in vitro* experiments, pregnant WT and KO mice were randomly assigned to different treatment groups and injected intraperitoneally with 10 mg/kg or 20 mg/kg PIC on E12.5. Control mice received saline injection (100 µl) at the same time point. Primary hippocampal cells were obtained from embryos of both genotypes from control and PIC-treated animals on E17.5–E18.5 and processed as described previously for morphological studies.

Then, possible inflammatory changes in the MIA model with the higher dose of PIC (20mg/kg) was measured. IL-1β levels were measured 24 hours after the saline and PIC injections in the fetal brain from E13.5 embryos with the quantitative analysis of IL-1β using enzyme-linked immunoassay sandwich technique (ELISA).

For behavioural studies, offspring of either control or immune-activated animals were weaned into cages of 2-4 animals at P21. Behavioural experiments were performed on test-naive male mice at P60-P90 in the same order (open field, T maze, novel object recognition, social preference and prepulse inhibition test) by an experimenter blinded to the treatments (Fig. 2).

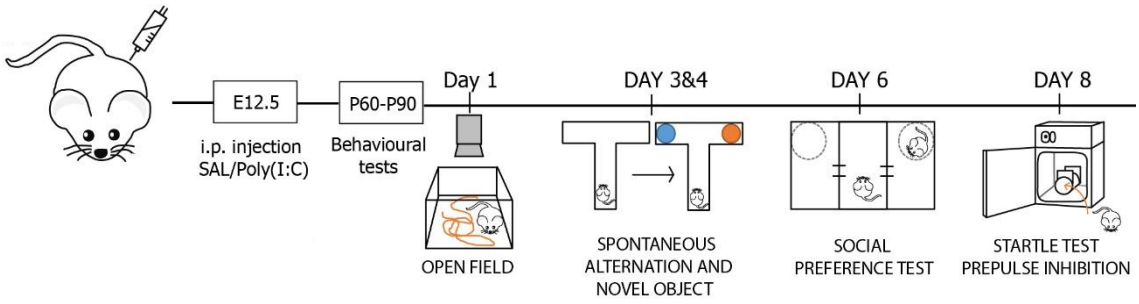

Figure 2. Battery of behavioural tests.

4. Conclusions

The following conclusions can be drawn from the studies performed regarding the physiological studies:

1. Higher transient expression of P2X7R is necessary for normal dendritic outgrowth during neuronal development, proliferation, and maturation, and its downregulation after the first stages of development relegates the receptor to pathological conditions in the adulthood.
2. Morphological and synaptic deficits are cell-specific in the hippocampus.
3. Behavioural deficits related to cognitive performance are present at the youngest stages but not in the adulthood, showing a slower maturation process in P2X7R-deficient mice.

The following conclusions can be drawn from the studies performed regarding pathological studies:

1. In a PCP model, P2X7R endogenous activation elicits social withdrawal performance and hyperactivity.
2. Endogenous activation of the receptor in a MIA model compromises dendritic outgrowth *in vitro* and might contribute to developmental and cognitive behavioural deficits in a mouse model of schizophrenia instead.
3. Neuroinflammation in mice during the second trimester of pregnancy partially reproduces the symptoms of schizophrenia observed in young patients, possibly mediated by the endogenous activation of P2X7R during the inflammatory insult. Therefore, this highlights the translational potential of P2X7R as a therapeutic target for schizophrenia.

Therefore, P2X7R has different regulatory functions depending on the stage of maturation (development or young adulthood) and whether receptor activation occurs. Additionally, activated P2X7R appears to have a pivotal role during pathological inflammatory events in immune activation models such as the MIA model, driving schizophrenia-like behaviours.

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