STUDY OF THE EFFECT OF P2X7 RECEPTOR PROTEIN FUNCTIONAL EXPRESSION IN THE PHENCYCLIDINE MURINE MODELS FOR SCHIZOPHRENIA

PhD thesis

Stefano Calovi

János Szentágothai Doctoral School of Neurosciences

Semmelweis University

Supervisor: Beáta Sperlágh, MD, PhD, D.Sc

Official reviewers:

Head of the Complex Examination Committee:

Members of the Complex Examination Committee:

Budapest
2021

INTRODUCTION

Schizophrenia is a complex psychiatric condition of which etiology, neurological substrate and pathological basis are still largely uncovered. Available treatments, although bearing risks of important side effects, can manage positive symptoms, yet lack efficacy for symptoms regarding cognitive dysfunctions. Preclinical studies implying PCP systemic administrations in rodents offer the possibility to observe, in healthy wild-type individuals, quantifiable behaviours related to the whole schizophrenia spectrum symptomatology.

P2X7Rs are protein channels abundantly expressed in the brain, mainly silent and considered to participate principally in immuno-pathological contexts. Empirical evidence demonstrated that loss-of-function of P2X7R exerts protective effects in several neurological disease model. Yet, the P2X7R mode of action and molecular mechanisms in models of psychosis remain unclear, and indirect evidence points to different directions.

The following work contributes to answering some questions regarding these apparently independent systems, the pharmacological PCP mouse models for schizophrenia and the ATP ionotropic receptor PX7R. We will conclude that, in mice, detrimental symptoms of schizophrenia are modulated by the functional presence of the P2X7R protein. Curiously, P2X7R is more related to immune and glial functions than to classical synaptic neurotransmission, the place of action of PCP and psychotomimetic drugs. Inhibitors of P2X7R, typically lowering the inflammatory profile in various systems, have shown repeatedly a good level of safety but have never been tested on schizophrenic patients, making worth the study about potential benefits.

Preclinical research of purinergic signaling in psychiatric models.

From the late 90s, the murine models for schizophrenia involving the dissociative anesthetic PCP have been greatly exploited, providing a basic understanding of the PCP-induced schizophrenia-like actions. Beáta Sperlágh, the supervisor of the current thesis, is a pioneering researcher of the purinergic signaling system neurobiology, internationally recognised for contributing to the study of the brain purinergic signaling, and especially the P2X7R protein, within pathological scenarios of mouse models for psychiatric disorders or conditions. The purinergic receptor P2X7 was here studied in the contexts of schizophrenia PCP-models over a mouse line genetically lacking the receptor's full-length expression ($P2rx7^{-/-}$). Indications in the literature that P2X7R loss-of-function lowers the effect of amphetamines was suggestive of some P2X7R-related interactions with psychotomimetic drugs. The first study using 2 and 5 mg/kg PCP on mice treated with P2X7R antagonists or genetically modified for PX7R shed lights on a possible protective effect of P2X7R loss-of-function. This study, published by our laboratory, found several adjustments at

the transcriptional level of neurotransmitter receptor proteins in the brain, which were associated to the P2X7R loss-of-function, and partly recovering the acute PCP detrimental effect.

In the current work we employed an approach placing behavioural measurements at the starting point of theory development. The employed biological models were wild-type C57Bl/6 mice and mice of the same strain, but or lacking the functional expression of the full-length protein (Pfizer knockout P2rx7$^{-/-}$) or overexpressing the functional protein fused with a fluorescent green protein (heterozygous P2rx7$^{tg/+}$). The PCP models for schizophrenia in rodents are considered solid tools to reproduce arrays of positive, negative and cognitive symptoms of schizophrenia. For this study the PCP dosage, treatment schedule, and animal's age were optimized to induce a working memory damage with repetitive administration, related to schizophrenia cognitive symptoms. Since only male subjects were employed, the current study is limited to be potentially predictive for male subjects.

The research about P2X7R

P2X7R is part of a ubiquitous and complex signaling system, the purinergic signaling system. Adenosine 5'- triphosphate (ATP) is the basic energy exchange molecule, synthesized by mitochondria in all eukaryotic cells, and present at millimolar concentration in the cytosol. ATP is very inefficient in diffusing across the cellular membrane, remaining highly compartmentalized inside the cells. In the nervous system, ATP is stored as a co-transmitter in all nerve types, and in physiological conditions, extracellular freely diffusing ATP is kept at concentrations in the low-micromolar range. ATP molecules get released in response to physiological and pathological stimuli, via exocytosis and transporter-mediated release. Thanks to an efficient neutralizing action, carried out by concerted metabolising and transporter proteins, intact tissue's parenchyma can reach maximal low-micromolar transient gradients of extracellular ATP. A second pathway through which ATP pours into the extracellular milieu is direct membrane leakage, common in case of injured tissues, dying cells and inflammation. In such pathological cases, a local surge of extracellular purines can transiently reach low-millimolar concentrations. Extracellular ATP and related metabolites exert pharmacological actions on a large family of receptors, called purinergic receptors, composing an entire intercellular communication system, highly conserved from an evolutionary point of view.

Purinergic receptors are divided into three subfamilies: P2X receptors (7 subunits) are trimeric ionotropic channels, P2Y receptors (8 subunits) and P1 receptors (4 subunits) are both G protein-coupled metabotropic receptors. P2X receptors activate exclusively upon ATP-binding, sensitive from nanomolar to low micromolar ATP-concentrations, with the P2X7R as an outlier, sensitive to hundreds of micromolar ATP. P2XR affinities for ATP combine with different desensitization

rates, with P2X7R often exhibiting complete lack of desensitization. P2XR ligand-binding opens a nonselective pore permeable to Na^+, K^+, and Ca^{2+} cations. Moreover, some P2X subunits, including P2X7R, provide an additional pathway for the passage of large organic cations.

Depending on the composition and location, P2X receptors exert different physiological actions, ranging from synaptic transmission, contraction of smooth muscle, secretion of chemical transmitters and regulation of the immune responses. The shape of the P2X7R is described as three "dolphin-like" subunits, collectively resembling a chalice, with the base in the membrane and the cup extracellular. The extracellular ATP-binding pockets are at the interface between subunits. Upon binding to ATP, the ion channel opens by conformational changes of the intracellular lower part of the receptor. The function of P2X7R was shown to be allosterically modulated by divalent cations as Mg^{2+}, Ca^{2+} and Zn^{2+}, steroids and lipids. The P2X7R is the longest P2XR with an exceptionally extended C-terminal tail. An interesting property that still raises scientific debates, is the so-called P2X7R-triggered "macropore" formation. In addition to the persistent current upon sustained stimulation, millimolar ATP concentrations typically induces aqueous pores on the cell membrane permeable to molecules up to 900 Da, constituted by auxiliary channels, and leading to the disruption of the ionic homeostasis of the cell. It has been observed that the $P2rx7^{-/-}$ Pfizer line used in the current study is associated with a minimization of such P2X7R aqueous pores formation.

P2X7R activation is a propeller of inflammation in the immune and nervous systems. The idea that blockade of P2X7R could hinder basic inflammatory processes in the brain introduced several P2X7R antagonists and P2rx7 genetic models to researchers all over the world. The brain is almost totally isolated from the systemic immunity, and neuroinflammation is essentially described as the innate immunological activation involving inflammatory-mediators signaling and microglia cell-type**.** P2X7R antagonists displayed protection in preclinical models with obvious neuroinflammatory components as pain-models, multiple sclerosis, cerebral ischemia etc., but showed as well efficacy in models presenting subtler neuroinflammation as depression, anxiety and bipolar disorder. Several P2X7R antagonists are proceeding through tens of clinical trials, usually showing good tolerability and encouraging efficacy. For the current study, considering our experimental models' complexity, we limited our experiments on P2rx7 genetic models.

In the CNS the P2X7R outlines the membrane of both microglial and oligodendroglia-lineage cells. As for neuronal expression, several pieces of indirect evidence were provided, yet a proof beyond doubt is still missing. We noticed in immunostaining pictures of the $P2rx7^{tg/+}$ mouse cortex the presence of P2X7R-EGFP "punctated" background signal, resembling typical synaptic protein immunostaining pictures. The $P2rx7^{tg/+}$ mouse line appears a powerful tool in future studies aiming to localize P2X7Rs in brain cells.

Microglia and extracellular ATP

Microglia is highly involved by purinergic signaling, *in vivo* and *ex vivo* constitutively expressing P2X7R. Yet, its expression level is dependent on unknown factors, even between very similar pathological contexts. Microglial P2X7R plays a crucial pro-inflammatory role as the second stimulus of a two-hit model. The first hit consists of signaling by immunologically active molecules that induces extracellular gradients of hundreds-micromolar ATP. This acutely inflamed tissue activates P2X7R channels, in turn leading to several interleukin release, with a cascade that, if not neutralized, initiates cytotoxic events. The P2X7R intracellular tail is estimated to interact with at least twenty proteins involved in immunological functions. The P2X7R persistent activation and "macropores" formation have been considered essential for the pro-inflammatory function, yet, the P2X7R-induced cytolytic death leaves behind a damaged and inflammogenic environment.

Microglia, the principal immune-competent cell-type in the CNS, guides immunological responses in acute phases, while keeping to readjust to homeostatic levels the brain, in physiolgical conditions. Microglial cells are sensitive to the subtlest changes in the chemical environment. For example, the mechanism guiding microglial branches towards gradients of increasing ATP concentrations (damage-seeking) depends on the P2Y12R activation, a purinergic metabotrobic receptor sensitive to micromolar ADP gradients. The impact of extracellular ATP on microglial function ranges from chemotaxis to death-induction. P2XRs activation induce transient depolarizing currents while P2YRs initiates delayed K^+ hyperpolarizing currents, both ending with an overall increase in the intracellular Ca^{2+} concentration. Microglial P2YRs are fundamental in damage-sensing chemotaxis, phagocytosis and surveillance, and are better understood than the P2XR-related one. On the other hand, P2XRs have been extensively investigated in relation with broader immunological functions, like disease models. The microglial, like every, P2X7Rs are sensitive to high concentrations of ATP and should remain silent in physiological conditions.

Modeling schizophrenia in mice: the effects of phencyclidine (PCP)

Schizophrenia is an emblematic psychiatric disorder characterized by the unpredictable young-adulthood onset of a pattern of abnormalities in thought and cognition, with a prevalence of 0.5% to 1% of the human population. Schizophrenia is diagnosed upon repeated psychotic episodes, commonly accompanied by a subtle decline of social and cognitive abilities**.** The most prominent neurological feature gets detectable only in advanced phases of the disease, consisting in cortical shrinkage and deterioration of key brain areas. While the etiology and patho-physiology of schizophrenia are far from being completely understood, few mechanisms are thought to be shared among a spectrum of different psychiatric conditions. Neurodevelopmental disorders as

schizophrenia and austism spectrum result from combinations of individual genetic/epigenetic susceptibility and environmental factors. In dedicated scientific literature, is observable redundancy in pointing to early development aspects, like dysfunctional parenting, complications in pregnancy and birth, head traumas, etc. Traditionally, physicians categorize schizophrenia into positive and negative symptoms. Positive symptoms include hallucinations, delusions, disorganized thoughts and movement, and are displayed during acute phases called psychotic episodes. The negative symptoms include emotional blunting, reduced sensation of pleasure, social withdrawal and cognitive deficits. Cognitive deficits are currently recognized as the most deteriorating and concealed aspects of schizophrenia, often referred as a third "cognitive" symptoms category, deteriorating functions like working memory, verbal learning and memory, attention, problem solving and social cognition. With the diagnosis for schizophrenia on solely psychiatric testing and the absence of any biomedical measurement, crediting relevant rodent models for schizophrenia is exceptionally challenging. Research on schizophrenia showed that both dopaminergic agonists and glutamatergic (NMDA-R) antagonists, like PCP, induce acute psychotic-like episodes in healthy volunteers, reproduce severe psychosis in schizophrenic patients treated with sub-effective doses, and is similarly effective in other mammals like primates and rodents. Sub-anesthetic PCP in humans commonly produce a state characterized by hallucinations, delusions, disorganized thought and speech, thereby mimicking several schizophrenia positive symptoms. Moreover, a behavioral and cognitive downfall, similar to negative and cognitive symptoms of schizophrenia, regularly escalates with the repetitive use of the substance. The employed PCP-model aims to mimic schizophrenia-like chained psychotic episodes via repeated PCP in young adult mice, when idiopathic schizophrenia is diagnosed and prior to the closure of frontal brain development.

The identification of the "PCP-receptor" as the excitatory NMDA-R initiated the study of PCP-like drugs in rodents. The NMDA-R are abundantly distributed in glutamatergic synapses throughout all the nervous system. The NMDA-R mechanism of action is responsible for multisynaptic neuro-transmission, synaptic plasticity, memory consolidation, etc. In the last 30 years the rodent PCP models became a standard to study schizophrenia, superseding former amphetamine models. The long-lasting negative- and cognitive-like effects after chronic PCP suggest a connection between schizophrenia and NMDA-R hypofunctionality. The brain regions most influenced by both PCP and schizophrenia symptoms are the limbic system, the sensory and frontal cortices and the monoaminergic mesencephalic nuclei. PCP was often adopted to test the efficacy of already approved as well as novel antipsychotic medications in preclinical studies. At sub-anesthetic ranges PCP acts as a specific and potent non-competitive antagonist of the NMDA-R. Arylcyclohexylamines, the dissociative anesthetic drugs derived from PCP, are use-dependent channel blockers, acting only when the pore is open. This characteristic gave them appeal for

pathologies with important components of excitotoxicity, like epilepsy and ischemia. The glutamate-binding NR2 subunit antagonism of PCP, however, besides limiting neuronal hyper-activity promotes local apoptosis and neurodegeneration, excluding the clinical applicability.

The current state of the art says that we cannot say with certainty and details how systemic PCP affects the brain or why it induces this “schizophrenic” behaviors. But decades of research are mapping the brain activity of all areas and cell types, linking synaptic functions with psychotomimetic behavior, in both rodents and humans. Some analogies between post-mortem molecular studies of schizophrenia and PCP action, the similar phenotypic and behavioral outcome are proof in support of the model's translational validity.

The lipophilic PCP cross the blood-brain barrier with extraordinary efficiency, getting stored dose-dependently in adipose tissues. This may be interesting considering that negative- and cognitive-like deficits after chronic PCP are documented days and weeks after its last administration. The acute psychotic-like PCP effects may either sensitize or desensitize with the repetition of administrations. At the end of 7-15 daily injections rodents start presenting a broader spectrum of symptoms, including negative- and cognitive-like symptoms.

To my knowledge, the first hypothesis for a general PCP mechanism consisted in the reduction of excitatory synaptic activity involving distal regions at the same time, like cortical primary sensory areas, and partly explains hallucination and delusional states. The theoretical point of PCP-models consists exactly in the abundance and ubiquitous expression of its target, a major difference respect to amphetamines- or LSD-based models. Different and not mutually exclusive hypothesis on PCP and schizophrenia models involve monoaminergic mechanisms, cortical inhibitory interneurons mechanisms, primary thalamic versus prefrontal disinhibition mechanisms, among others. Therefore PCP-models may depend on the summation of small effects, which synergistically converge into the prototypical phenotype. For example, PCP induces frontal dopaminergic release by stimulating the firing of VTA neurons; this mechanism explains quite specifically the addictive property of PCP-like anesthetics. PCP at high concentrations presents several off-targets, influencing also the dopaminergic signaling systems, but within the “psychotomimetic” doses PCP affects exclusively NMDA-R.

The current work focused on the prefrontal cortex (PFC), more precisely the medial PFC of the mouse (mPFC), an associative area coordinating cognitive processes, regulation of emotion, motivation, and sociability. Lesions in the mPFC are associated with impairments found in essentially every neurological and psychiatric model with cognitive dysfunctions. The mPFC of rodents is often compared to the dorsolateral prefrontal cortex of the human's brain. Working memory is defined as the temporary storage, filtering and computation of sensory stimuli no

longer existing. The mPFC integrity and engagement is considered crucial to carry out this continuous update of information and adaptation of thoughts and behaviors into the changing environment. Working memory is frequently damaged in schizophrenic patients, and greatly responsible for general poor outcome of schizophrenic patients. Most importantly, working memory deficits in schizophrenia are resistant to any currently available treatment.

In rodents the mPFC undergoes pronounced enhancement of neuronal activity following systemic PCP delivery, to which corresponds behavioral expressions of positive-like symptoms, while chronic PCP consistently disrupts working and episodic memory. Pyramidal neurons contribute to 80-90% of the total mPFC neurons, and the remaining 10-20% is represented by inhibitory GABAergic interneurons. Dorso-ventral differences of laminar organization and connectivity divide the mPFC into agranular cingulate cortex (ACC), prelimbic cortex (PL), infralimbic cortex (IL). The inputs to the circuit are predominantly received by the superficial layer II/III, and the main output are the projections from the layer V large pyramidal neurons. Wide-distribution systems, like dopaminergic and serotonergic networks, exert complex influential modulations of the overall mPFC activity. Typical and atypical antipsychotics show high affinity for monoamine receptors, *e.g.* chlorpromazine to D2R or *e.g.* clozapine to serotonergic 5-$HT_{2A/2C}$, 5-HT_{1A} receptors and α-adrenoceptors, and some speculate to primarily target the PFC. Beyond the shrinkage of the PFC recurrently observed in advanced stages of schizophrenia, a subtler decrease in density of GABAergic parvalbumin (PV) or somatostatin interneurons are the second most reported events in human studies, phenomenon that is replicated with PCP mouse models.

Systemic PCP in anesthetized rats modulates the mPFC activity via activation of thalamic efferent fibers, resulting in a longitudinal band of c-Fos positive neuronal nuclei between the layers III to VI. The centromedial and mediodorsal thalamocortical neurons connected to the mPFC also gets hyperactivated by systemic PCP. Apparently, only systemic PCP administration evokes glutamate and monoamine level increase in the PFC area, since topical application results mostly as local electrical activity silencing.

In young adult mice acutely treated with 10 mg/kg, PCP produces a transient neurochemical imbalance that profoundly alters the whole physiological brain activity, resulting into expression of positive-like symptoms behaviours. With repetitive PCP administrations the accumulation of the psychotomimetic plus withdrawal experiences results in few specific brain damages, while the behaviour presents long-lasting abnormalities.

Immunological responses during pregnancy are described as risk factors for schizophrenia. Simulations of viral infection during rodents pregnancy is an alternative validated preclinical model for schizophrenia and autism. Moreover, high levels of pro-inflammatory markers inside

and outside the schizophrenic CNS are other indications of immunological or inflammatory events in this psychiatric condition. White matter abnormalities, axonal degeneration and active neuroinflammation were successfully identified in schizophrenic patients. A recent trend to explore the influence of "neuroinflammation and glial cells" in schizophrenia is developing in the scientific community. Hampering the shift of microglia towards proinflammatory phenotype via pharmacological intervention is one of the most accredited between these novel hypotheses. Yet, the literature present limited evidence on the existence of microglial activation after subchronic treatment, even with high PCP doses.

P2rx7$^{-/-}$ mice, besides encompassing an anti-depression phenotype, revealed resistance against acute amphetamine-induced hyperactivity. The hypothesis proposed at the time was a central P2X7R-dependent mechanism which reinforces the activation of the stress axis (hypothalamus-pituitary-adrenal). After extensive research on the role of P2X7R in mood disorders, recently was hypothesized that ATP, released by astrocytes, activates the microglial P2X7R, triggering a neuroinflammation that can potentially govern several aspects of the disease. The P2X7R loss-of-function in models of schizophrenia as well could, in theory, restrict the slow progression of a neuroinflammatory phenomenon. Few studies also suggested a direct action of some antipsychotic drugs on *e.g.* ATP-evoked P2XR responses, or *e.g.* negative allosteric modulation of P2X7R.

In 2016 our laboratory published the first paper finding P2X7R genetic deletion or pharmacological blockade alleviates the acute psychotomimetic effects of PCP, employing the low-doses of 2 and 5 mg/kg. These changes were accompanied by alterations of the expression of schizophrenia-related genes in the PFC. In the current study, we investigated the role of P2X7R functional expression in acute and subchronic PCP-models of schizophrenia, using animals lacking (P2rx7$^{-/-}$) or overexpressing (P2rx7$^{tg/+}$) the full-length P2X7R.

AIMS:

- Establish a pharmacological mouse model of positive and cognitive symptoms of schizophrenia by employing acute and subchronic delivery of PCP.
- Identify possible differences regarding PCP-related behaviors between the P2rx7 genetically modified strains, and investigate the possible correlation with molecular and neurological measurements in the mouse mPFC.
- Investigate the possibility of involvement of neuroinflammatory phenomena in the PCP model in relation to P2X7R expression in the mouse mPFC.
- Examine the role of local P2X7R functional expression in native conditions at the level of the mouse mPFC.

RESULTS:

To test the validity of the P2rx7$^{-/-}$ model in the frontal cortical area, RT-qPCR experiments were performed to detect P2rx7 gene transcripts. Measurements confirmed that the knocked-out sequence was absent in P2rx7$^{-/-}$ mice and expressed by P2rx7$^{+/+}$ mice.

Results: Animals littermates and unrelated: 5 WT, 5 KO (p60-70). Knocked-out sequence (relative expression): P2rx7$^{+/+}$ 1,12 ± 0,12, P2rx7$^{-/-}$ 0,02 ± 0,04; unpaired Student's *t-test* *p*-value < 0.01, in collaboration with Pàl Tod.

To evaluate the involvement of P2X7R in the PCP-induced hyperlocomotor activity, P2rx7$^{+/+}$ and P2rx7$^{-/-}$ mice were treated with vehicle or 10 mg/kg PCP *i.p.* and submitted to a long open-field test, using a novel cage as an arena. PCP in all subjects increased the locomotion and stereotypical behaviors, such as head weaving and rotational walking. The PCP-induced hyperactivity peaked between 40 and 80 minutes from the delivery, and PCP-treated P2rx7$^{+/+}$ animals exhibited more stereotypy and a longer-lasting hyperlocomotion respect to P2rx7$^{-/-}$ PCP-treated mice.

Results: Animals littermates and unrelated: 4 WT and KO SAL, 5 KO PCP, 6 WT PCP (p65-78). Average velocity, 80 to 100 minutes (cm/s): WT PCP 6,0 ± 1,7, KO PCP 3,1 ± 0,3; two-way ANOVA and Bonferroni's *posthoc* test *p*-value < 0.01. Average stereotipycal behavior, 40 to 80 minutes (rotations numbers): WT PCP 237 ± 38, KO PCP 178 ± 16; two-way ANOVA and Bonferroni's *posthoc* test *p*-value < 0.05.

Acute systemic PCP induces hyperactivity of the mPFC circuit. Neuronal activation was monitored using c-Fos immunohistochemistry in combination with the behavioral protocol. The c-Fos expression, that correlates with the single neuron's activity, is an established molecular marker of recently active neurons. Along the mPFC of PCP-treated animals, appeared the aforementioned band of c-Fos immuno-positive neuronal somata (c-Fos^{+}), concentrated around the upper layer V, and fading along the dorsal mPFC. In P2rx7$^{-/-}$ mPFC, a lower concentration of PCP-induced c-Fos^{+} nuclei in the layer II/III of ventral mPFC was detected at first.

Results: Animals littermates and unrelated: 3 WT and KO SAL, 4 WT and KO PCP (p65-78). Quantification c-Fos^{+} neurons, layer II/III, ventral mPFC (cells/mm^2); SAL < 20; WT PCP 69 ± 13; KO PCP 27 ± 14; two-way ANOVA and Bonferroni's *posthoc* test *p*-value < 0.05 (WT PCP vs. WT SAL), < 0.01 (WT PCP vs. KO SAL), KO PCP vs SAL not significant.

Considering the nuclear levels of c-Fos to be proportional to the neuronal activity, we performed a deeper analysis in PL and IL areas of interest of the mPFC. Pragmatic discrimination between activated (c-Fos^{+}) and strongly activated (here referred as c-Fos^{++}) neurons enabled to set the

parameters for semi-automated quantification. A significant decrease in the concentration of c-Fos^{++} neurons was detected in the layer V of IL and PL areas in P2rx7$^{-/-}$ mice.

Results: Animals littermates and unrelated: 4 WT and KO PCP (p65-78). Quantification c-Fos^{++} neurons (intensity threshold), layer V, PL and IL mPFC (cells/mm^2); DAB: WT PCP 237 ± 21, KO PCP 121 ± 7; Fluo: WT PCP 271 ± 14, KO PCP 142 ± 27; two-way ANOVA and Bonferroni's *posthoc* test *p*-value < 0.005 (DAB), <0,01 (Fluo).

Therefore, psychotomimetic effects of 10 mg/kg PCP upon acute behavior and mPFC layer-specific neuronal activation was partially buffered by the genetic deletion of P2rx7$^{-/-}$.

PV interneurons are proposed to be determinants in both the PCP and schizophrenia underlying mechanisms. Double immunohistochemistry for c-Fos and PV neuronal markers were performed in the mPFC of acutely PCP-treated P2rx7$^{+/+}$ and P2rx7$^{-/-}$ mice. No difference was found between the tested genotypes, neither in the total number of PV positive interneurons, nor in percentages of PV-immunopositive cells activated during the systemic PCP effect, at the mPFC layers II/III and V.

Results: Animals littermates and unrelated: 4 WT and KO PCP (p65-78). Manual counting of PV$^+$ interneurons concentrations, layer V, centro-dorsal mPFC (cells/mm^2); WT PCP 73 ± 11, KO PCP 61 ± 9; Active PV$^+$ interneurons, layer V, centro-dorsal mPFC (%), WT PCP 16 ± 2, KO PCP 11 ± 2; two-way ANOVA and Bonferroni's *posthoc* test, not significant.

We then moved our focus on the dopaminergic influence. No significant genotype-related anatomical differences were observed in immunostainings for monoaminergic fibers (Tyrosine hydroxilase) in the mPFC.

Preliminary data: animals littermates and unrelated, 2 per group (p65-78). Longitudinal intensity profiles, mPFC TH/c-Fos double ihc, two-way ANOVA and Bonferroni's *posthoc* test, not significant (TH); *p*-value < 0.05 for WT PCP vs. KO PCP, 270-280 μm depth from the layer I surface (c-Fos).

Therefore we studied the properties of dopamine release fibers in the PFC of untreated animals. The veratridine stimulation, inducing Na^+ channel-mediated dopamine release, did not differ between wild-type and P2rx7$^{-/-}$ or P2rx7$^{tg/+}$ animals. The PFC of P2rx7$^{-/-}$ animals displayed lower basal dopamine release, confirming our previous finding.

Results: Animals littermates and unrelated: 14 WT and 8 KO (p60-90). ^{3}H-Dopamine fractional release (%); WT 1,41 ± 0,08, KO 1,04 ± 0,06; unpaired Student's *t-test p*-value < 0.001.

On the other hand, the uptake of dopamine found in P2rx7$^{tg/+}$ PFC was lower when compared to the corresponding wild-type controls.

Results: Animals littermates and unrelated: 4 WT and 8 TG/+ (p59-68). ^{3}H-Dopamine tissue uptake (kBq/g); WT 368 ± 12, TG/+ 221 ± 21; unpaired Student's *t-test* *p*-value < 0.05. In collaboration with E.S. Vizi laboratory.

After typical PCP-related topics, we addressed the role of the P2X7R expressed by microglial cells in the context of PCP-driven mPFC activation in P2rx7$^{-/-}$ mice. No differences in microglial density and general ramification were identified between groups, while the P2Y12R immune-reactivity lowered in PCP-treated P2rx7$^{-/-}$, if compared with P2rx7$^{+/+}$ treated mice.

Results: Animals littermates and unrelated: 3 WT and KO SAL, 4 WT and KO PCP (p65-78). P2Y12R ihc. mean grey value (a.u.); WT PCP 794 ± 13, KO PCP 702 ± 21; P2Y12R stained area (%); WT PCP 20,4 ± 0,5, KO PCP 17,5 ± 1,1; two-way ANOVA and Bonferroni's *posthoc* test *p*-value < 0.05 (mean grey value); < 0.01 (stained area).

Regions of interests like IL and PL underwent a soma-centered 2D-Sholl analysis on non-isolated microglial cells, an atypical method, first proposed by Calovi et al., 2020. It provides information about microglia morphological complexity, and the readout is a mixed profile, with the small radius describing a single cell ramification, and larger radius invading a more and more inter-cellular territory, patrolled by several microglial branches. The single-cell ramifications resulted similar between all groups, but in the intercellular territory, the saline-treated P2rx7$^{-/-}$ microglial processes resulted significantly denser respect to the P2rx7$^{+/+}$ ones. Interestingly this difference faded following PCP.

Results: Animals littermates and unrelated: 3 WT and KO SAL, 4 WT and KO PCP (p65-78). 2D cell-centers analyzed (numbers): 38 WT SAL, 48 KO SAL, 60 WT PCP, 59 KO PCP. Number of crossing branches per radius – profile (curve) Mann-Whitney U-test, radius 0-20 μm not significant; radius 20-40 μm *p*-value < 0.005 KO SAL vs. WT SAL; *p*-value < 0.01 KO SAL vs. WT PCP.

Another 2D Sholl analysis of microglial branches was this time centered on PCP-activated or inactivated neuronal nuclei. In this way, we can estimate the number of microglial processes organized around hyperactivated neuronal nuclei. Neurons not involved in the PCP hyperactivation were found less surrounded by microglial branches in both the genotypes, in the PL and IL layer V areas.

Results: Animals littermates and unrelated: 4 WT and KO PCP (p65-78). 2D cell-centers analyzed (numbers): 52 WT NeuN, 51 KO NeuN, 34 WT c-Fos, 36 KO c-Fos. Number of crossing branches per radius, profile (curve) two-way ANOVA and Bonferroni's *posthoc* test *p*-value = 0,0265 in 8 μm radius (WT), < 0,0001 in 15 μm radius (KO).

We also noticed that strongly activated neurons in P2rx7$^{-/-}$ tissue had the tendency to be in contact with microglial cell body. So, a more rigorous 3D Sholl analysis of microglial branches centered on c-Fos^{+} nuclei, eventually found a small but significant increase in number of branches recruited by the PCP-activated neurons in the P2rx7$^{-/-}$ cortex.

Results: Animals littermates and unrelated: 3 WT and KO PCP (p65-78). 3D cell-centers analyzed (numbers): 52 WT PCP, 45 KO PCP. Number of crossing branches per radius, profile (curve) Mann-Whitney U-test p-value < 0.005.

Moving on, patch-clamp electrophysiology in acute slices was performed to investigate a possible congenital/developmental intrinsic hypoexcitability of the P2rx7$^{-/-}$ pyramidal neurons. Thirteen neurons from 8 P2rx7$^{+/+}$ animals and twenty-one neurons from 6 P2rx7$^{-/-}$ animals mice older than 59 days were recorded and analyzed. No differences in series resistance (35.7 ± 3.2 MΩ P2rx7$^{+/+}$; 33.4 ± 2.4 MΩ P2rx7$^{-/-}$) and membrane resistance (375.6 ± 38.7 MΩ P2rx7$^{+/+}$; 433.5 ± 56 MΩ P2rx7$^{-/-}$) were found between genotypes. Similarly, no difference was found in resting membrane potential (mV, -76,1 ± 4,5 P2rx7$^{+/+}$; -79,5 ± 1,6 P2rx7$^{-/-}$), rheobase (pA, 78,2 ± 8,3 P2rx7$^{+/+}$; -83,8 ± 11,6 P2rx7$^{-/-}$) or the resting-voltage/current relationship in a 20 pA current steps protocol. Following action potential firing, the P2rx7$^{-/-}$ mPFC neurons displayed faster repolarization of the membrane potential respect to P2rx7$^{+/+}$ neurons, which fired a higher number of action potentials per equivalent current pulse, suggesting a difference in spike accommodation.

Results: Animals mixed: 8 P2rx7$^{+/+}$ (12 neurons) and 6 P2rx7$^{-/-}$ (19 neurons). Action potentials count during 400 ms I_{step}, Mann-Whitney U-test p-values < 0.05. In collaboration with Jan Tønnesen.

The electrophysiological data suggest that mPFC layer V pyramidal neurons from P2rx7$^{+/+}$ mice respond more robustly to membrane depolarization than P2rx7$^{-/-}$, consistent with previous c-Fos results and fitting with dopamine and microglia measurements, and pointing towards the loss-of-function of P2X7R as a protective mechanism against acute behavioral and neurological psychotomimetic PCP effects.

To corroborate these findings we adopted a recently developed C57Bl/6J mouse line overexpressing the P2X7R-EGFP protein (P2rx7$^{tg/+}$), kindly provided by Prof. Annette Nicke. We confirmed via immunostainings the presence of the P2X7R-EGFP protein expression in the mPFC, mainly concentrated over microglial and oligodendroglial membranes.

To assess the susceptibility to acute PCP, P2rx7$^{tg/+}$ and P2rx7$^{+/+}$ mice received a 2 mg/kg low-dose PCP, followed by a “coupled open-field” and social withdrawal protocol. Basal levels of locomotion, social behavior, stereotypical behavior, and ataxia were not different between saline-treated animals. In P2rx7$^{+/+}$, acute PCP treatment strongly diminished the social interaction, and

promoted stereotypical behavior. P2rx7$^{tg/+}$ mice, beyond disrupted sociability and stereotypy, exhibited hyperlocomotion and features of ataxia in a few individuals.

Results: Animals littermates and unrelated: 10 WT SAL, 12 TG/+ SAL, 6 WT PCP, 12 TG/+ PCP (p57-71). Locomotory activity (LINEX): WT PCP 408 ± 41, TG/+ PCP 582 ± 15; Ataxia score (Log): WT PCP 0,005 ± 0,004, TG/+ PCP 0,066 ± 0,028; one-way ANOVA and Dunn's *posthoc* test *p*-value < 0,01 (locomotion), Kruskal-Wallis test *p*-value < 0,05 (ataxia). Collaboration with Paula Mut-Arbona.

The 10 mg/kg acute PCP experiment was repeated testing the P2rx7$^{tg/+}$ mouse line. The psychotomimetic effects of PCP resulted exacerbated in P2rx7$^{tg/+}$ animals, particularly in terms of stereotypy, respect to the P2rx7$^{+/+}$ mice.

Results: Animals littermates and unrelated: 3 WT and TG/+ SAL, 9 WT and TG/+ PCP (p65-75). Average velocity, 30-70 minutes (cm/s): SAL < 3; WT PCP 5,0 ± 0,3, TG/+ PCP 6,3 ± 0,2; two-way ANOVA and Bonferroni's *posthoc* test *p*-value < 0.05 SAL vs. WT PCP; < 0.005 SAL vs. TG/+ PCP. Average stereotipycal behavior, 30 to 70 minutes (rotations numbers): SAL < 50, WT PCP 211 ± 35, TG/+ PCP 341 ± 34; two-way ANOVA and Bonferroni's *posthoc* test *p*-value < 0.01 SAL vs. PCP; < 0,005 WT PCP vs. TG/+ PCP.

Fluorescent c-Fos immunostainings and analysis of IL and PL layer V area pictures revealed a higher concentration of strongly activated (c-Fos^{++}) positive nuclei, at bregma values of +1.70 but not +2,10, in the PCP-treated P2rx7$^{tg/+}$ brains.

Results: Animals littermates and unrelated: 4 WT and TG/+ PCP (p65-75). Quantification c-Fos^{++} neurons (intensity threshold), layer V, PL and IL mPFC (cells/mm^2); bregma +1,7: WT PCP 167 ± 19, TG/+ PCP 271 ± 15; bregma +2,1: WT PCP 173 ± 23, KO PCP 202 ± 27; unpaired Student's *t-test p*-value < 0.01 bregma +1,7: not significant bregma +2,1. Collaboration with Paula Mut-Arbona and Andras Iring.

Therefore, respect to acute PCP, P2X7R overexpressing mice present the opposite profile respect to the P2rx7$^{-/-}$ mice genetically encoding a loss-of-function protein, aggravating the behavioral expression of positive-like symptoms and increasing layer-specific activation of the mPFC.

We proceeded to evaluate the impact of cognitive-like symptoms induced by seven consecutive PCP systemic injections, using single-caged P2rx7$^{-/-}$ and P2rx7$^{+/+}$ animals. Before treatments, P2rx7$^{-/-}$ animals had a higher body mass compared to the P2rx7$^{+/+}$ animals, which normalized 24 hours from the first injection.

Results: Animals littermates and unrelated, 8 WT and KO SAL, 12 WT and KO PCP. Average weight during PCP subchronic treatment (gr.) day 1 WT 25,7 ± 1,5, KO 29,2 ± 0,9, day 2 WT 26,5 ± 1,1, KO 28,3 ± 1,0; two-way ANOVA and Bonferroni's *posthoc* test *p*-value < 0.05 (day 1), not significant (day 2).

Once finished the PCP treatment plus 72 additional hours of withdrawal, mice were tested in a y-maze to assess the status of their working memory. In an ethologically relevant paradigm, the successful spontaneous alternations test, $P2rx7^{+/+}$ PCP-treated mice displayed poorer working memory performances respect to the $P2rx7^{+/+}$ saline- and $P2rx7^{-/-}$ PCP-treated animals.

Results: Animals littermates and unrelated, 12 WT and KO SAL, 12 WT PCP, 14 KO PCP (p60-67). Successful alternations (%): WT SAL 62,4 ± 2,1, KO SAL 59,9 ± 3,0, WT PCP 52,5 ± 3,2, KO PCP 66,1 ± 3,5; unpaired Student's *t*-test *p*-value < 0.05 WT SAL vs WT PCP, < 0.05 KO PCP vs WT PCP.

Considering that neuroinflammatory events are accompanied by changes in cytokine concentrations, we quantified brain levels of inflammatory mediators reportedly dependent on P2X7R-mediated mechanisms. At the 7th day of treatment withdrawal, the retrosplenial cortex and the PFC were extracted, homogenized and quantified. While no differences were found between groups in the retrosplenial cortex, a significant increase in the fractalkine ligand (CX3CL1) levels was found in the $P2rx7^{-/-}$ PFC, after both PCP and vehicle subchronic treatments. All other measured cytokines levels appeared not affected by neither the treatment nor the genotype, with many measures close to the detection limit of the technique.

Results: Animals littermates and unrelated, 8 per group (p60-67). Cx3Cl1 quantification with bead-array CBA analysis, from PFC (pg/mg): WT SAL 2,1 ± 0,4, KO SAL 10,1 ± 2,4, WT PCP 1,9 ± 0,5, KO PCP 7,6 ± 4,2, two-way ANOVA and Bonferroni's *posthoc* test *p*-value < 0.05 WT vs KO PCP; < 0.01 WT vs. KO SAL.

The results of subchronic PCP experiments, beyond confirming the absence of a PCP-related smoldering neuroinflammation, suggest a beneficial role for P2X7R loss-of-function in the induced working memory deficit, a major symptom of schizophrenia still currently untreatable and well mimicked in PCP models.

Eventually, a few additional unpublished data are concluding the dissertation's results. For example, during the optimization of the subchronic behavioral paradigms we realized that the PCP-induced working memory deficit, as we measured it in a y-maze, was not detectable in animals housed in group-cages during the treatment, but only in single-caged mice. Moreover, single-caged animals performed higher numbers of same arm repeated entries respect to group-caged animals.

Results: Animals littermates and unrelated, single-cage, 12 WT and KO SAL, 12 WT PCP, 14 KO PCP; group-caged 13 WT SAL, 7 KO SAL, 14 WT PCP, KO PCP (p60-80). Spontaneous successful alternations (%): single-WT PCP 52,5 ± 3,2, grouped-WT PCP 61,2 ± 2,8, single-KO PCP 66,1 ± 3,5, grouped-KO PCP 65,5 ± 3,0; Same arm entries repetitions (numbers): single-WT PCP 156 ± 31, grouped-WT PCP 34 ± 2,

single-KO PCP 58 ± 7, grouped-KO PCP 26 ± 5, two-way ANOVA and Bonferroni's *post-hoc* test not significant (% s.a.), *p*-value < 0,05 KO PCP; < 0,005 WT PCP (same arm entries).

We observed also weather subchronic PCP treatment induced long-lasting effects via two 15-minutes open-field tests in large arenas, performed 10 and 11 days from the last injection. All animals displayed a normal behavior, with the only subtle increase in PCP-treated $P2rx7^{+/+}$ animals spontaneous rotations, significant against PCP-treated $P2rx7^{-/-}$ mice, that normalized the following day. All animals were significantly less active the second test, habituating to the paradigm as normal.

Results: Animals littermates and unrelated, washout-day 10: 8 WT SAL, 8 KO SAL, 9 WT PCP, 10 KO PCP; washout-day 11: 8 WT SAL, 8 KO SAL, 9 WT PCP, 9 KO PCP. Spontaneous circular rotations, day 10 (number): WT SAL 38 ± 4, KO SAL 37 ± 3, WT PCP 44 ± 4, KO PCP 33 ± 5; one-way ANOVA and Bonferroni's *post-hoc* test *p*-value <0,05 WT PCP vs. KO PCP (day 10).

To explore neurological substrates of the subchronic PCP effect, a pilot study on wild-type animals attempted to identify general "PCP molecular fingerprints" in the mPFC. PV immunostaining analysis, with our PCP model, could not detect the PCP-induced decrement of PV cortical expression. Pictures of c-Fos immunostainings for quantifying mPFC basal activity presented a very low signal. Despite using a Matlab automated macro specific for c-Fos counting, $c\text{-}Fos^{+}$ concentration resulted low and no differences were found.

Results: Animals littermates and unrelated, 5 WT SAL and 5 WT PCP (p60-70). PV^{+} interneurons count after subchronic treatments, layer V (cell/mm^2): SAL 5,9 ± 0,4, PCP 7,1 ± 0,8; two-way ANOVA and Bonferroni's *post-hoc* test, not significant. PV mean signal intensity of mPFC layers II-V (a.u.): SAL 51,3 ± 9,7, PCP 65,2 ± 11,3; unpaired Student's *t-test,* not significant. Automated quantification of $c\text{-}Fos^{+}$ in mPFC, layer III (cell/mm^2): SAL 155 ± 16, PCP 107 ± 21, one-way ANOVA and Bonferroni's *post-hoc* test, not significant.

Glial components seemed also interesting for histological analysis of subchronically treated $P2rx7^{+/+}$ and $P2rx7^{-/-}$ mice, sacrificed 4 days from the last treatment. Iba-1 stainings and whole-PFC pictures were semi-automatically quantified, resulting in Iba-1signal weakening in the PCP-treated $P2rx7^{-/-}$ PFC.

Results: Animals littermates and unrelated: 3 WT and KO SAL; 4 WT and KO PCP, 3-5 pictures per animal (p60-70). Iba-1 nuclei automatic quantification, medial and orbital cortex (cell/mm^2): WT SAL 362 ± 32, KO SAL 474 ± 63, WT PCP 436 ± 35, KO PCP 268 ± 41; two-way ANOVA and Bonferroni's *post-hoc* test *p*-value < 0.005 KO PCP vs. KO SAL, < 0.005 KO PCP vs. WT PCP.

P2Y12R immunostainings of layer II-VI in the PL cortex did not reveal any difference in microglial cell density. Eventually, the GFAP staining was generally weak in the whole PFC, and low numbers of positive cells makes statistics unreliable.

DISCUSSION:

The main finding of the present dissertation is that the effect of PCP in mice is partly modulated by the P2X7R functional expression, in terms of positive and cognitive symptoms. $P2rx7^{-/-}$ animals were less susceptible to the psychotomimetic effects of the dissociative anesthetic, while the overexpression of the purinergic receptor produced a positive-like symptom response, within a suboptimal dose for PCP-induced psychosis-like activity, and exacerbated stereotypy with a psychotomimetic PCP dose. Moreover, after a subchronic PCP treatment, $P2rx7^{-/-}$ mice did not display the typical impairment in a y-maze working memory task. Observed behaviors correlated with histological data obtained from the brain of the tested animals. We found an alleviation of acute PCP-driven neuronal activation in a restricted area of the mPFC (layer V of PL and IL areas) in $P2rx7^{-/-}$ mice, while higher involvement of mPFC circuit was recorded with the receptor overexpression.

Acute 10 mg/kg PCP treatment in C57Bl/6 mice resulted in a high number of heavily stained c-Fos neuronal nuclei, an indirect measurement of *in vivo* neuronal activity. Comparing the same area, $P2rx7^{-/-}$ and $P2rx7^{tg/+}$ mice displayed a decreased and increased concentration of strongly activated neurons, respectively. Considering the histological data, the [^{3}H]-DA release experiments and electrophysiological characterizations, the overall profile seems consistent with a pleiotropic role for the P2rx7 gene, which functionally seemed to increase the mPFC microcircuit activity. We also observed subtle but interesting changes in the microglia/neuron contacts at the level of PL and IL layer V, suggestive of P2X7R participation in such interactions. With the available data we should not speculate further conclusions. This PCP study confirms and extends our and others observations obtained with P2X7R-genetic deletion and -pharmacological inhibitors.

For the first time we challenged $P2rx7^{tg/+}$ young-adult animals with PCP psychotomimetic effects. The $P2rx7^{tg/+}$ high-sensitivity to PCP, in terms of behaviour and mPFC neuronal hyperactivation, adds relevance to the PCP model, as schizophrenic patients also display increased response to arylcyclohexylamines anesthetics. The lower dopamine uptake of $P2rx7^{tg/+}$ animals may indicate prefrontal hyperdopaminergia, a feature converging with many types of psychosis.

To summarize the PCP-acute effects study, the functional expression of P2rx7 full-length protein resulted positively correlated with the level of manifestation of the symptoms and the related mPFC neuronal activity.

The treatments of cognitive deficits represent the most unmet clinical need concerning schizophrenia. We examined the working memory of P2rx7$^{-/-}$ mice following a subchronic PCP regimen that compromises memory performances. With the beginning of the treatment faded the genotypical difference in animal's weight, normally present and supposedly deriving from alternative metabolic pathways. After 7 days of treatment and 3 of withdrawal, animals were tested for the spontaneous alterations test in a y-maze. Subchronic PCP significantly impaired the working memory performance of wild-type mice respect to vehicle- and P2rx7$^{-/-}$ PCP-treated groups. The cytokines quantification did not reveal any neuro-inflammatory process relative to PCP. Interestingly, the prefrontal level of fractalkine or Cx3Cl1, an essential trophic factor involved in microglial physiology, was strongly upregulated in P2rx7$^{-/-}$ brains.

In summary, central P2X7R function is able to modulate the positive- and cognitive-like symptoms induced by PCP in mice. PCP treatment acutely triggers the stress response, the hypothalamus-pituitary-adrenal (HPA) axis, by stimulating pituitary adrenocorticotropic hormone release and the consequent increase of plasma corticosterone levels. An interesting possibility is that P2rx7$^{-/-}$ animals' behaviour, which naturally display an "anti-depressed" profile, could depend from a lowered centrally-dependent HPA axis activation. The constitutionally anti-stressed state could logically evolve in an anti-depressed phenotype P2rx7 loss-of-function dependent.

CONCLUSION:

In the current work we collected evidence of different nature, from behavioural observation to electrophysiological characterization of prefrontal neurons, trying to evaluate the effect of the ATP purinergic P2X7R functional expression in the PCP murine schizophrenia-model. In line with previously published results, we found that P2X7R loss-of-function confers a protective phenotype for what concerns PCP psychotomimetic effects, both in terms of acute psychotic-like behaviour (positive-like symptoms) and of subchronic PCP-derived working memory dysfunction (cognitive-like symptoms). We corroborated our observations by the study of a P2X7R overexpressing mouse line that presented exacerbated PCP-induced positive-like symptoms. Although we could not perform direct experiments interrogating the specific molecular mechanisms and cell types involved in P2X7R function, collectively, our findings support the notion that full-length P2X7R expression, probably via multiple roles, exacerbate the pathological effects of PCP, while the absence of it partly suppresses both positive and cognitive-like symptoms. To conclude we find encouraging the revealed efficacy of the P2X7R loss-of-function in both categories of positive- and cognitive-like symptoms, which in the future we hope will get corroborated, for example, with P2X7R-antagonist studies in similar models.

List of publications
Publications related to this thesis:

Original research articles:

Koványi B[1], Csölle C, Calovi S, Hanuska A, Kató E, Köles L, Bhattacharya A, Haller J, Sperlágh B. (2016) *The role of P2X7 receptors in a rodent PCP-induced schizophrenia model.* **Scientific Reports** Nov 8;6:36680.

Calovi S[1], Mut-Arbona P, Tod P, Iring A, Nicke A, Mato S, Vizi ES, Tønnesen J, Sperlagh B. (2020) *P2X7 Receptor-Dependent Layer-Specific Changes in Neuron-Microglia Reactivity in the Prefrontal Cortex of a Phencyclidine Induced Mouse Model of Schizophrenia.* **Frontiers in Molecular Neuroscience.** Nov 11;13:566251.

Review published in the context of the PhD studies:

Calovi S[1], Mut-Arbona P, Sperlágh B. (2019) *Microglia and the Purinergic Signaling System.* **Neuroscience**. May 1;405:137-147.