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Investigating the Roles of PLPPR3 in Central Nervous System Function and Behavior

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Abstract

Phospholipid phosphatase-related proteins (PLPPRs) are a family of brain-enriched membrane proteins implicated in axonal development, synaptic plasticity, and neurobehavioral regulation. Among them, PLPPR3 has recently emerged as a key player in neuronal morphogenesis, yet its in vivo role in behavioral regulation and intracellular signaling remains largely unexplored. This thesis investigates the behavioral and molecular consequences of PLPPR3 deletion in mice, aiming to elucidate its involvement in anxiety-related behaviors and signaling pathways. To this end, PLPPR3 knockout (KO) and wild-type (WT) C57Bl/6 mice were subjected to a battery of behavioral tests, including the Open Field Test (OFT), Elevated Plus Maze (EPM), Marble Burying Test (MBT), and acute restraint stress protocols. Pharmacological challenges were also introduced using amphetamine, a psychostimulant that enhances dopaminergic transmission and activates cAMP-PKA signaling, and PF-8380, an autotaxin inhibitor that reduces lysophosphatidic acid (LPA) levels. Behaviorally, PLPPR3 KO mice exhibited increased locomotor activity and reduced anxiety-like behaviors in the OFT and EPM, along with decreased compulsive-like behavior in the MBT. These effects were attenuated following acute stress, which induced a robust anxiogenic response in both WT and KO mice. Pharmacologically, amphetamine administration induced hyperlocomotion independent of genotype, while PF-8380 treatment failed to modulate anxiety-like behavior. At the molecular level, analyses of the prefrontal cortex and hippocampal synaptosomes did not reveal alterations in signaling cascades associated with Akt-GSK3, ERK-CREB in basal and amphetamine treated conditions, with the exception of increased mTOR pathways as suggested by increased pS235/236-S6 in PLPPR3 KO PFC. Collectively, these findings support a modulatory role of PLPPR3 in anxiety-related behavior, highlighting a possible interface between PLPPR3 function, bioactive lipid signaling, and dopaminergic neurotransmission and offer new insights into the molecular underpinnings of emotional behavior and psychiatric vulnerability.

Περίληψη

Οι πρωτεΐνες που σχετίζονται με τις φωσφολιπιδικές φωσφατάσες (PLPPRs) αποτελούν μια οικογένεια μεμβρανικών πρωτεϊνών με σημαντική παρουσία στον εγκέφαλο, οι οποίες εμπλέκονται στην ανάπτυξη των αξόνων, τη συναπτική πλαστικότητα και τη νευρο-συμπεριφορική ρύθμιση. Μεταξύ αυτών, η PLPPR3 (επίσης γνωστή ως PRG2) αναδείχθηκε πρόσφατα ως βασικός παράγοντας στη νευρωνική μορφογένεση, ωστόσο ο in vivo ρόλος της στη ρύθμιση της συμπεριφοράς και στην ενδοκυτταρική σηματοδότηση παραμένει σε μεγάλο βαθμό ανεξερεύνητος. Αυτή η διατριβή διερευνά τις συμπεριφορικές και μοριακές συνέπειες της έλλειψης PLPPR3 σε μυς, με στόχο να διαλευκάνει τη συμμετοχή της σε συμπεριφορές που σχετίζονται με το άγχος και σε οδούς σηματοδότησης. Για τον σκοπό αυτό, μυς PLPPR3 knockout (KO) και άγριου τύπου (WT) C57Bl/6 υποβλήθηκαν σε μια σειρά συμπεριφορικών δοκιμασιών, συμπεριλαμβανομένης της δοκιμασίας ανοιχτού πεδίου (OFT), της δοκιμασίας υπερυψωμένου σταυροειδούς λαβυρίνθου (EPM), της δοκιμασίας εναπόθεσης σβώλου (MBT) και πρωτοκόλλων στρες. Εφαρμόστηκαν επίσης φαρμακολογικές παρεμβάσεις χρησιμοποιώντας αμφεταμίνη, ένα ψυχοδιεγερτικο που ενισχύει τη ντοπαμινεργική σηματοδότηση και ενεργοποιεί τη σηματοδότηση cAMP-PKA, και PF-8380, έναν αναστολέα της αυτοταξίνης που μειώνει τα επίπεδα του λυσοφωσφατιδικού οξέος (LPA). Συμπεριφορικά, τα ποντίκια PLPPR3 KO εμφάνισαν αυξημένη κινητικότητα και μειωμένες συμπεριφορές άγχους στις δοκιμασίες OFT και EPM και MBT. Αυτά τα αποτελέσματα μετριάστηκαν με την παρουσία στρες, η οποία προκάλεσε μια ισχυρή αγχώδης απόκριση τόσο στους μυς WT όσο και στους KO. Φαρμακολογικά, η χορήγηση αμφεταμίνης προκάλεσε υπερκινητικότητα, ανεξάρτητη από τον γονότυπο, ενώ η θεραπεία με PF-8380 δεν κατάφερε να ρυθμίσει τις συμπεριφορές που σχετίζονται με το άγχος. Σε μοριακό επίπεδο, οι αναλύσεις του προμετωπιαίου φλοιού και των συναπτοσωμάτων απο ιππόκαμπο δεν αποκάλυψαν διαφοροποιήσεις στις οδούς σηματοδότησης που σχετίζονται με τις οδούς Akt-GSK3, ERK-CREB τόσο σε κανονικές συνθήκες όσο και σε δείγματα από μυς μετα απο χορήγηση αμφεταμίνης με την εξαίρεση της αύξησης της σηματοδότησης του mTOR και την αυξημένη pS235/236-S6 στον PFC PLPPR3 KO μυών. Συλλογικά, αυτά τα ευρήματα υποστηρίζουν έναν ρυθμιστικό ρόλο της PLPPR3 σε συμπεριφορές που σχετίζονται με το άγχος, υπογραμμίζοντας μια πιθανή διασύνδεση μεταξύ της λειτουργίας της PLPPR3, της σηματοδότησης των βιοδραστικών λιπιδίων και της ντοπαμινεργικής σηματοδότησης, και προσφέρουν νέες γνώσεις για τις μοριακές βάσεις της συναισθηματικής συμπεριφοράς και των ψυχιατρικών διαταραχών.

Abbreviations

AC: Adenylyl cyclase
ADHA: Attention deficit hyperactivity
AKAPs: A-kinase anchoring proteins
Akt: Protein kinase B
AMPH: Amphetamine
ASD: Autism spectrum disorder
ATX: Autotaxin
BASP1: Brain Abundant Membrane Attached Signal Protein 1
cAMP: cyclic AMP
CNS: Central nervous system
CREB: cAMP response element-binding protein
CSF: Cerebrospinal fluid
DAT: Dopamine transporters
EPM: Elevated Plus Maze
ERK: Extracellular Signal-Regulated Kinases
ERM: Ezrin/radixin/moesin
GSK3: Glycogen Synthase Kinase 3
KO: Knockout
LPA: Lysophosphatidic acid
LPC: Lysophosphatidylcholine
LTD: Long term depression
LTP: Long term potentiation
MAG: Monoacylglycerol
MAPK: Mitogen-activated protein kinase
MBT: Marble burying test
mTOR: mammalian target of rapamycin
NET: Norepinephrine Transporter
OFT: Open Field Test
PI3K: Phosphoinositide 3-kinase
PIP3: Phosphatidylinositol (3,4,5)-trisphosphate
PKA: Protein kinase A
PLPPs: Phospholipid phosphatases
PLPPRs: Phospholipid phosphatase-related proteins
PRGs: Plasticity-related genes
PSD95: Postsynaptic density protein 95
PTEN: Phosphatase and tensin homolog
Raf: Rapidly Accelerated Fibrosarcoma
Ras: Rat sarcoma virus

RasGRF1: Ras protein-specific guanine nucleotide-releasing factor 1
RhoA: Ras homolog family member A
ROCK: Rho Associated Coiled-Coil Containing Protein Kinase
SAL: Saline
SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SERT: Serotonin transporter
TAAR1: Trace Amine-Associated Receptor 1
TBS: Tris Buffered Saline
TBE: Tris-Borate-EDTA
WT: Wild-type

1. Introduction

1.1. PLPPR proteins: key players in neuronal development and synaptic function

The development and adaptability of the brain rely on a wide variety of signaling molecules that work together, both extrinsic or intrinsic, to guide how neurons grow, connect and communicate through synapses (Roelfsema & Holtmaat, 2018). Recent studies have highlighted the role of a class of membrane proteins known as phospholipid phosphatase-related proteins (PLPPRs), also referred to as plasticity-related genes (PRGs), that have emerged as regulators of filopodia formation, axonal growth, synaptic transmission and excitability in the central nervous system (CNS) (Fuchs et al., 2022; Polyzou et al., 2024).

PLPPRs comprise a family of five evolutionarily conserved transmembrane proteins (PLPPR1-5), derived from the broader lipid phosphatase/phosphotransferase family (Polyzou et al., 2024). Structurally, PLPPRs contain six transmembrane domains, with both N- and C-termini oriented intracellularly. However, unlike classical phospholipid phosphatases (PLPPs), PLPPRs are catalytically inactive due to amino acid substitutions in the canonical catalytic motifs in the extracellular domain (Fuchs et al., 2022) (Figure 1.1). Despite this, they retain the capacity to modulate bioactive lipid signaling, particularly through interactions with lysophosphatidic acid (LPA) signaling pathways (Fuchs et al., 2022; Polyzou et al., n.d.; Vogt et al., 2016).

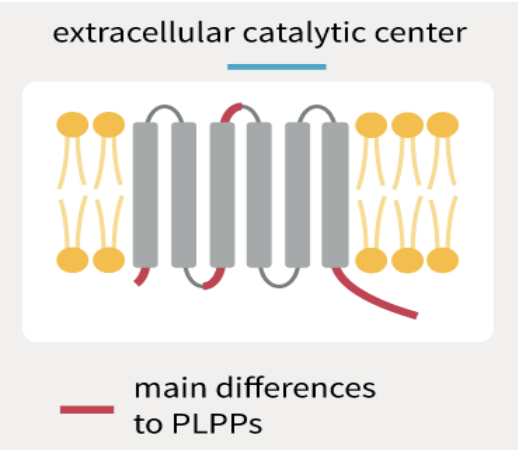

Figure 1.1 General structure of PLPPRs. Shown are the 6-transmembrane domain regions and regions that differentiate from related PLPPs. Note that PLPPR3 and PLPPR4 have a large intracellular C-terminal domain (not shown in the Figure). Putative LPA-interacting residues are located in extracellular loops 2 and 3 (Fuchs et al., 2022).

PLPPRs display temporally and spatially regulated expression patterns in the brain that suggest distinct roles during neurodevelopment and neural circuit maturation (Figure 1.2A). During embryonic and early postnatal stages, PLPPRs are robustly expressed, particularly in regions undergoing active neurite outgrowth and synaptogenesis. This developmental peak aligns with periods of intense structural remodeling in the nervous system. As the brain

matures, expression levels of these proteins tend to decrease globally. Certain isoforms, however, retain enriched expression in adult brain regions such as the hippocampus, cerebral cortex, cerebellum, and olfactory bulb, implicating their involvement in maintaining plasticity in specific circuits (Fuchs et al., 2022). Interestingly, PLPPRs seem to differentiate in terms of temporal expression patterns; for example, PLPPR2 and 4 exhibit robust expression in late developmental stages and in the adult cortex and hippocampus, while PLPPR1, 3 and 5 expression peaks at earlier developmental stages (Fuchs et al., 2022; Gross et al., 2022). Nevertheless, detailed RNA and protein expression analyses have shown that earlier expressed PLPPR1 and PLPPR3 exhibit ensuing expression in specific adult brain regions such as the nucleus accumbens and striatum (Figure 2B) (Polyzou et al., 2024).

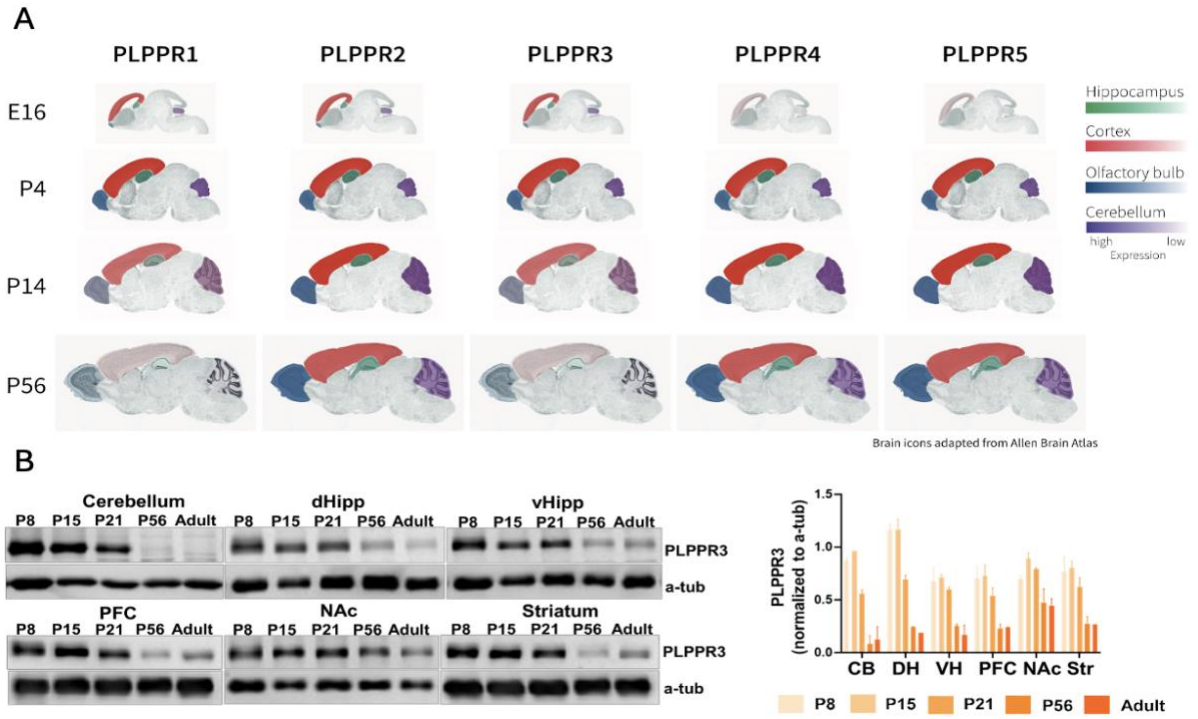

Figure 1.2 A. The expression pattern of PLPPRs in the rodent brain in developmental stages and adult brain. Colours and colour intensity depict the specific regions PLPPRs are localized and differentially expressed (Fuchs et al., 2022), B. PLPPR3 protein expression in developing and adult brain regions of Wistar rats (Polyzou et al., 2024).

Functionally, one of the most conserved and well-documented cellular roles of PLPPRs is the promotion of filopodia-like membrane protrusions (Fuchs et al., 2022). Overexpression studies in cultured neurons and non-neuronal cell lines have shown that PLPPR1, 3, 4, and 5 drive the formation of actin-rich protrusions reminiscent of filopodia, which are essential precursors to neurite initiation, axonal branching, and dendritic spine formation (Leondaritis & Eickholt, 2015) (Figure 1.3). These structures are critical for establishing neuronal polarity and facilitating contact with guidance cues and synaptic partners. The filopodia-inducing activity is primarily attributed to the intracellular C-terminal domain of PLPPRs. However, the

specific molecular interactions remain incompletely characterized and it has been proposed that PLPPRs may employ non-classical mechanisms for filopodia induction that may involve cytoskeletal dynamics through indirect or scaffold-like mechanisms, and modulation of lipid signaling or membrane curvature (Fuchs et al., 2022).

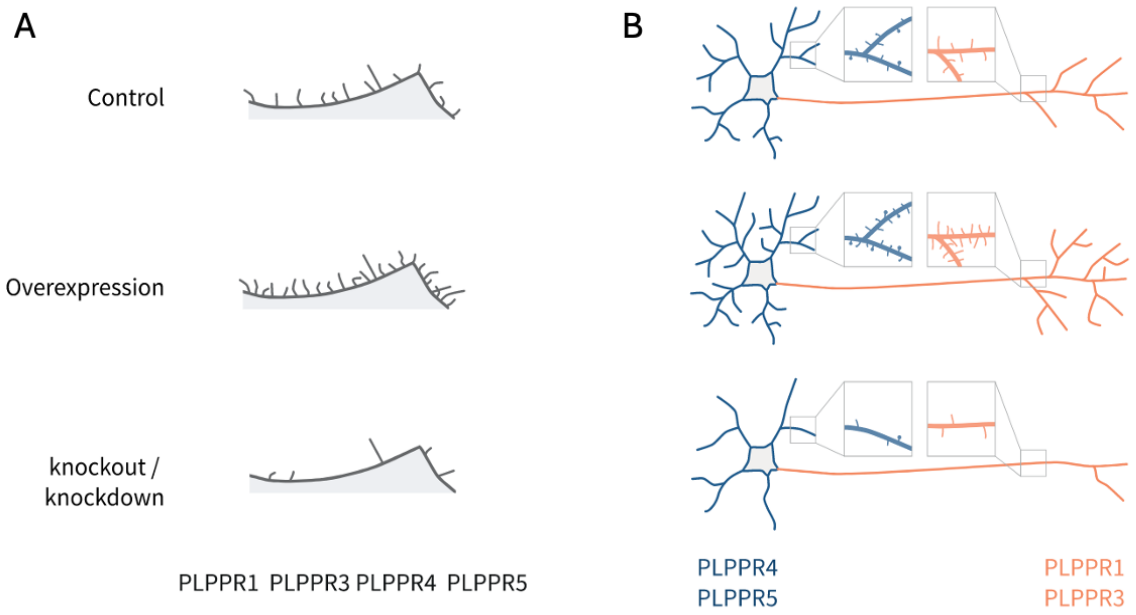

Figure 1.3 A. Filopodia formation in cultured cells related to PLPPRs expression. B. FIlopodia and branch formation and neuron morphology depending on PLPPRs expression. Blue color depicts dendrite morphology and orange color axon morphology (Fuchs et al., 2022)

1.2. PLPPRs as Putative Modulators of Lipid Signaling in the Brain

Another documented role of PLPPRs is their apparent ability to modulate lipid signaling in developing and mature neurons. Lipid signaling molecules have emerged as central regulators of neuronal development and plasticity (Tracey et al., 2021). LPA is a prominent extracellular bioactive phospholipid in the CNS. It acts as an endogenous agonist of at least six G protein-coupled receptors (LPAR1-6), activating downstream signaling pathways such as PI3K-Akt, RhoA-ROCK, and MAPK/ERK (Yanagida & Shimizu, 2023; Yung et al., 2015). These pathways orchestrate a broad range of biological processes including neuronal differentiation, axonal guidance, synaptic function and network excitability (Hernández-Araiza et al., 2018).

Extracellular LPA is mainly synthesized by autotaxin (ATX), a dedicated extracellular phospholipase D that hydrolyzes extracellular lysophospholipids such as lysophosphatidylcholine (LPC), and it is hydrolyzed and deactivated to monoacylglycerol (MAG) by PLPP ectophosphatases (Yung et al., 2015). Its effects can also be mediated by many non-canonical receptor-like plasma membrane proteins, including PLPPRs (Polyzou et al., 2026). Rather than hydrolyzing LPA, PLPPRs appear to modulate LPA responses by various alternative mechanisms, such as controlling local LPA concentrations, altering lipid access to

LPARs, or interacting with key intracellular LPA effectors (Fuchs et al., 2022; Polyzou et al., 2026). The proposed modes of interaction of PLPPRs with LPA signaling are depicted in Figure 1.4 and further analyzed for specific PLPPRs in the following paragraphs.

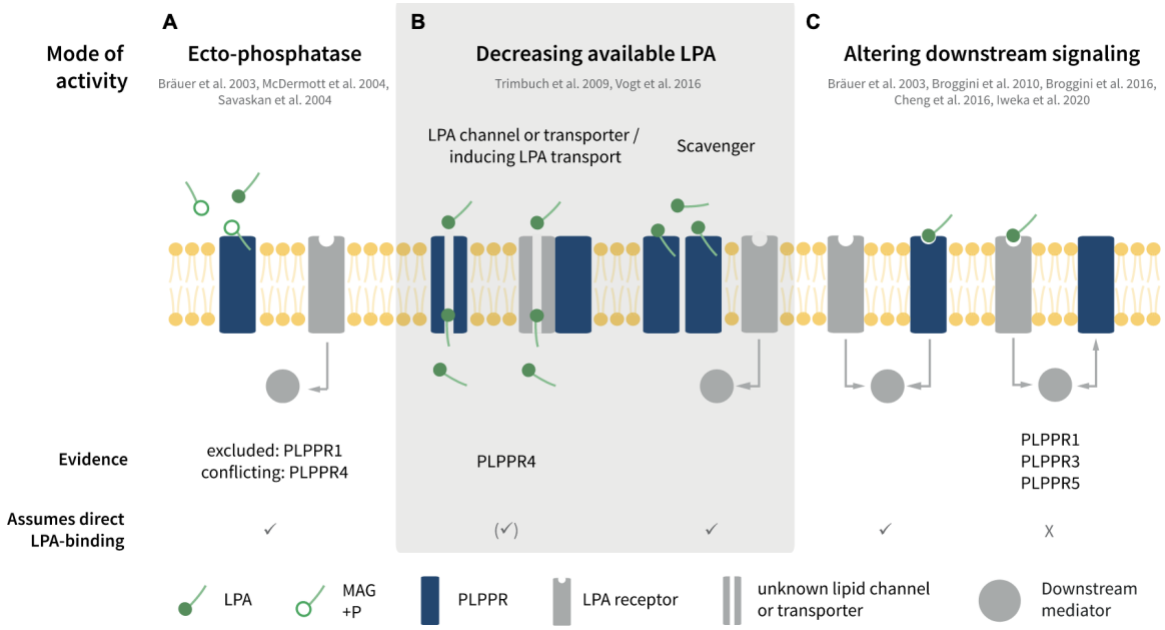

Figure 1.4 Proposed modes of interaction of PLPPRs with LPA. A. PLPPRs as ectophosphatases. B. PLPPRs as channels, transporters or scavengers. C. PLPPRs as downstream signaling mediators (Fuchs et al., 2022)

PLPPR1 (also known as PRG3 or LPPR1), is predominantly localized on the presynaptic compartments of neurons, especially within axons and growth cones (Fuchs et al., 2022). Functionally, it is involved in neuronal development, synaptic plasticity and regeneration (Brandt et al., 2024). PLPPR1 induces membrane protrusions through RasGRF1 (Broggini et al., 2016). Also, PLPPR1-expressing cells counteract the effect of LPA as a neurite growth inhibitor. PLPPR1 expression inhibits LPA-induced activation of RhoA and as a result it blocks the LPA-mediated neurite retraction (Broggini et al., 2016; Fan et al., 2016). In addition, PLPPR1 can interact with RhoGDI1, a negative regulator of Rho GTPases, further supporting its role in promoting axonal elongation (C. Agbaegbu Iweka et al., 2021).

Also localized in the axonal compartment, PLPPR3 (also known as PRG2 or LPPR3) is mainly localized to axonal filopodia and branches (Fuchs et al., 2022). PLPPR3 overexpression in cell cultures and stem cell derived neurons induced formation of filopodia and branches (Brosig et al., 2019; Fuchs et al., 2020), while PLPPR3 deficiency led to a lower number of axonal filopodia and eventually decreased axonal branch stability in primary hippocampal neurons (Brosig et al., 2019; Fuchs et al., 2022; Fuchs & Eickholt, 2021). PLPPR3 has been shown to enable LPA-induced changes in cellular and axonal morphology, such as cell rounding, neurite collapse, and reduction of axonal branches in early developed neurons in vitro (Polyzou, 2024). Accordingly, PLPPR3 has been linked to the LPA dependent guidance of thalamocortical

axons in vivo, via interaction with radixin (RDX), an ezrin/radixin/moesin (ERM) cytoskeleton-binding protein (Cheng et al., 2016).

PLPPR4 (also known as PRG1 or LPPR4) is the most-well studied member of the family. It is localized mainly at postsynaptic sites, particularly in dendritic spines of hippocampal neurons (Fuchs et al., 2022), playing a key role in regulating hippocampal excitability, promote axonal outgrowth and regeneration after brain injury (Bräuer et al., 2003; Trimbuch et al., 2009). The major proposed function of PLPPR4 is to act as an atypical LPA transporter at the synapse (Trimbuch et al., 2009; Vogt et al., 2016). Postsynaptic PLPPR4 removes LPA from the synaptic cleft, and thus it prevents LPA-induced activation of presynaptic LPAR2 receptors, suggesting a unique mechanism in which atypical and canonical LPA pathways converge at the synapse (Tokumitsu et al., 2010; Trimbuch et al., 2009; Vogt et al., 2016). Through this interaction, PLPPR4 modulates glutamatergic neurotransmission, potentially serving as a buffer against excitotoxicity, thereby stabilizing synaptic responses and protecting neurons. Independent of LPA, PLPPR4 has also a physiological role in spine density, long term potentiation (LTP) and memory formation (Liu et al., 2016).

These distinct patterns of function suggest that PLPPR1, 3 and 4 coordinate different aspects of neurodevelopmental and synaptic plasticity. During neurodevelopment, PLPPR1 and 3 appear essential for axonal guidance and elongation, while PLPPR4 plays a critical role in the maturation and function of the synapses. Together, they support the proper wiring and stabilization of neural circuits, partially through the modulation of LPA signaling with high importance for both developmental neuroscience and neurotherapeutics.

1.3. PLPPRs, LPA and Behavior: Emerging Evidence from KO Models

LPA is a powerful bioactive lipid that has been involved in several CNS pathologies. Many of the studies have utilized knockout strategies in mice to investigate the effects of specific LPAR deletion, centered around LPAR1 and LPAR2, in anxiety and psychiatric disorders, Alzheimer's, Parkinson's, multiple sclerosis, traumatic brain injury, spinal cord injury and pain (Dedoni et al., 2025). Deletion of LPAR1 in mice has been associated with various behavioral defects related to psychiatric diseases, such as schizophrenia, cognitive impairment, anxiety disorder, posttraumatic stress disorder, and substance abuse (Yanagida & Shimizu, 2023). A specific strain of LPAR1 KO mice, referred to as the maLPA1-null mice, represents a validated model of anxious depression. maLPA1-null mice present increased anxiety-like behavior, impaired spatial memory and reduced adaptive coping under stress (Moreno-Fernández et al., 2017; Santin et al., 2009). These mice also exhibit reduced hippocampal BDNF levels and lower rates of neurogenesis that correlate with poor cognitive flexibility and elevated limbic system activation. In addition, a mass spectrometry profiling of hippocampal tissue has shown that acute stress increases the levels of specific LPA species, particularly LPA 18:0 and 18:1. In

LPAR1 KO animals, these changes are enhanced, linking biochemical LPA imbalance to vulnerability to stress induced behavioral dysfunction (Tabbai et al., 2019). Apparently, the involvement of LPA signaling in anxiety and stress is multifactorial and complex, since intracerebroventricular LPA administration has been shown to induce anxiety-like behavior in mice, without affecting locomotor activity in a dose dependent experiment. These effects are attenuated by an LPAR1-4 antagonist suggesting a LPAR-mediated effect, at least partially (Yamada et al., 2015).

LPA has been identified as a potential factor in the development of schizophrenia, as LPA signaling can mimic schizophrenia-like behaviors and brain changes in animal models. In a prenatal schizophrenia-like animal model, LPA-LPAR1 were identified as key factors and modulators of the schizophrenia model. Prenatal injection of LPA induced schizophrenia-like behavior in adult animals. Moreover, prenatal injection of LPAR1 antagonists blocked the LPA induced schizophrenia-like phenotypes (Mirendil et al., 2015). Besides LPAR1, LPAR2 has also been suggested to impact on LPA-related CNS pathologies. In this case, however, the neuropsychiatric roles of LPAR2 are specifically dependent on PLPPR4. Thalman et al. demonstrated that the postsynaptic PLPPR4 acts as a synaptic brake on LPA signaling. Mice carrying a point mutation in PLPPR4 (R346T) had impaired LPA uptake, leading to increased synaptic LPA, hyperexcitable cortical activity and sensory gating deficits, including reduced prepulse inhibition (PPI). In addition, PLPPR4 KO presented an increased mean gamma coherence that is typically altered in patients with psychiatric disorders like schizophrenia. These behavioral impairments were rescued by autotaxin inhibition, linking PLPPR4 dysfunction directly to excessive LPA signaling and behavioral dysregulation (Thalman et al., 2018). This work establishes a regulatory circuit where astrocytic ATX generates extracellular LPA near the synapse, and the excessive LPA is cleared by PLPPR4 in the postsynaptic terminal, while LPAR2 modulates the presynaptic glutamate release. Dysregulation of this system results in neuronal network hyperexcitability, which is linked to psychiatric symptoms such as anxiety and psychosis. Altogether, LPA has been mentioned as a key factor for initiation of schizophrenia symptoms, while antagonizing its receptors or blocking the production can be a versatile strategy for treatment of psychiatric disorders.

Although LPAR2 KO mice are similar to WT mice in conventional learning and memory tests, they show faster and more accurate responses in tasks that require high attention and fast spatial discrimination. Particularly in aging mice, LPAR2 inhibition has been proposed as bearing therapeutic benefits to manipulate aging-associated cognitive disorders (Fischer et al., 2021).

Many studies have strengthened further the PLPPR4/LPAR2 connection. Overexcitation of cortical neurons, increased EPSC amplitude and epileptiform discharges in hippocampal networks were observed in PLPPR4 KO mice (Trimbuch et al., 2009). Importantly, in PLPPR4/LPAR2 double KO mice this hyperexcitability is reversed. Another PLPPR4 KO mice model exhibited hyperlocomotion, increased exploratory behavior and repetitive self-

grooming (Schneider et al., 2018), resembling endophenotypes of psychiatric disorders like autism and ADHD-like symptoms. Interestingly, these behaviors were not observed in PLPPR4/LPAR2 double KO, suggesting again that the behavioral phenotype is also LPAR2 dependent. Imaging and behavioral studies of PLPPR4 KO mice have suggested increased anxiety-like behavior, altered social interaction and impaired cognitive flexibility, phenotypes that are strongly connected to major psychiatric disorders (Endle et al., 2022; Schneider et al., 2018; Vogt et al., 2016). Alterations in the LPAR2-LPA-PLPPR4 axis have also been associated with food intake disruption. Fasting increases the synaptic LPA signaling, leading to hyperexcitability, increased exploration drive and fasting-induced hyperphagia, with ATX inhibition and LPAR2 deletion being able to normalize those phenotypes. As PLPPR4 is known to control LPA levels in the synapse, PLPPR4 deletion increased all the abovementioned phenotypes. Increased levels of LPA through PLPPR4 deletion also increased food intake and body weight in both animal and human models (Endle et al., 2022).

Although PLPPR4 is the most thoroughly studied isoform, there is growing recognition that PLPPR1, PLPPR3 and PLPPR5 are also functionally relevant to a range of behavioral phenotypes and neurological pathologies, although it is not still clear if all phenotypes depend on or relate to LPA signaling. PLPPR3 has been shown to be essential for proper thalamocortical axon guidance via LPA-mediated interaction with RDX (Cheng et al., 2016). While the study's focus was anatomical and electrophysiological, the disrupted thalamocortical targeting seen in PLPPR3 deficient mice implies downstream consequences for sensorimotor integration. Indeed, PLPPR3 KO mice have altered somatosensory cortical processing and a deficit in somatosensory discrimination (Cheng et al., 2016). For PLPPR1, behavioral studies are also limited but point toward its involvement in neuropsychiatric dysfunction. Deletion of PLPPR1 has been reported to cause hypoactivity, impaired sensorimotor gating and deficits in associative learning and memory, phenotypes that are characteristic of schizophrenia and autism, with LPA mentioned as possible interaction molecule (C. A. Agbaegbu Iweka, 2018). PLPPR5 has been connected to seizures and epileptogenesis, with PLPPR5 KO mice showing increased seizure latency, reflecting an increased brain excitability (D. Wang et al., 2021). In addition, in an animal model of juvenile seizures, PLPPR5 KO mice displayed neurobehavioral and cognitive impairments (D. Wang et al., 2022).

Altogether, these findings reinforce the idea that PLPPRs play crucial and divergent roles in shaping behavior, not only through structural development, but also through dynamic modulation of synaptic signaling and neuroplasticity. It should be stated however that, besides PLPPR4, there is no definite evidence of the involvement of LPA in many PLPPR-dependent behavioral phenotypes. PLPPR involvement in conditions ranging from epilepsy and cognitive delay to psychiatric vulnerability and motor dysfunction underscores their broad relevance in neurodevelopmental and neurodegenerative diseases.

1.4. Interaction of PLPPR3 with signaling pathways

1.4.1 PKA and PLPPR3 phosphorylation

Recent work has revealed that PLPPRs, and specifically PLPPR3, is controlled by various protein kinases via direct phosphorylation of several residues in its intracellular C-terminal domain (Kroon et al., 2024). Amongst these kinases, phosphorylation of PLPPR3 S351 by protein kinase A (PKA) has been thoroughly characterized to regulate the formation of a complex of PLPPR3 with the presynaptic protein brain abundant membrane attached signal protein 1 (BASP1), a known modulator of synaptic vesicle dynamics and actin organization. Interestingly, PKA-dependent PLPPR3 S351 phosphorylation is less robust in early development and does not control the filopodia and branch induction functions of PLPPR3, suggesting that it may be involved in other functions. Accordingly, PLPPR3 S351 phosphorylation has been shown to be confined in adult synaptosomal membranes and to regulate synaptic vesicle release in vitro. Deletion of PLPPR3 led to increased depolarization-induced synaptic vesicle release in cultured hippocampal neurons. This phenotype is corrected by expression of wild type PLPPR3, but not by a S351A PLPPR3 mutant that cannot be phosphorylated by PKA (Kroon et al., 2025, accepted manuscript). Importantly, this regulatory mechanism has been studied primarily in primary neuronal cultures and thus its physiological relevance in the in vivo setting is currently unknown. Nevertheless, it has been postulated that PLPRP3 in the adult presynapse may be under the control of synaptic PKA activity to fine-tune synaptic vesicle release (Kroon et al., 2024).

PKA is an important regulator of various neuronal processes in the developing and adult CNS, including synaptic plasticity, neuronal development and gene transcription (Glebov-McCloud et al., 2024). Biochemically, PKA is a serine/threonine kinase that is activated by cyclic AMP (cAMP). PKA's role in learning and memory is connected to its role in long-term potentiation (LTP) and long-term depression (LTD) (Glebov-McCloud et al., 2024). Its impact on memory is well understood with involvement in consolidation, retrieval and deletion of memory, through the cAPM-PKA-CREB pathway that affects both short-term and long-term memories (Glebov-McCloud et al., 2024; Vianna & Izquierdo, 2013). Also, it regulates Ca^{2+} influx through NMDA receptors, influencing synaptic plasticity that can vary depending on the developmental stage and the specific brain region (Yang et al., 2009). Variations in PKA are also connected to neurodevelopmental disorders that affect cognition, motor function and social behavior (Glebov-McCloud et al., 2024). PKA is also involved in other CNS functions including dopamine synthesis, mitochondrial dynamics and neuronal survival (Dagda & Das Banerjee, 2015). PKA is strategically localized at dendrites, dendritic spines, cytosol and axonal terminals through the scaffold proteins A-kinase anchoring proteins (AKAPs) (Glebov-McCloud et al., 2024).

PKA is modulated through various mechanisms, including changes in cAMP levels, interactions with regulatory subunits and phosphorylation of target proteins. It is under the control of

numerous neurotransmitter GPCR receptors that either activate or inhibit adenylyl cyclases (AC) and cAMP levels via coupling to Gs or Gi proteins. These GPCRs may be located either pre or postsynaptically (Glebov-McCloud et al., 2024). GPCRs control the activation of AC, which in turn is responsible for the production of cAMP. cAMP activates PKA unleashing the PKA catalytic subunit that either phosphorylate Ser/Thr residues on its substrates in the synapse and periphery or translocate to the soma and enter the nucleus targeting and phosphorylating nuclear factors, e.g. the cAMP response element-binding protein (CREB), a transcription factor involved in various processes including neurodevelopment and neuronal plasticity (H. Wang et al., 2018).

PKA interacts with the dopaminergic system in several ways. Dopamine receptor activation, particularly D1-type receptors (D1 and 5), are coupled to Gs proteins, which stimulate AC, leading to PKA activation, while D2-type receptors (D2, 3 and 4), are coupled to Gi/o proteins, that inhibit AC, decreasing cAMP levels and deactivating PKA (Mishra et al., 2018), dopaminergic system has been associated with major neurological diseases, with schizophrenia being one of the primary conditions discussed (Bhatia et al., 2025). A strong indirect agonist of the dopaminergic system is amphetamine. Amphetamine is a CNS stimulant that, under certain experimental conditions, functions primarily by increasing the amount of dopamine in the synaptic cleft via various mechanisms (Berman et al., 2009). It enters the presynaptic compartment through monoamine transporters DAT, NET and SERT and a) inhibits the vesicular monoamine transporter, b) disrupts the electrochemical gradient that is crucial for vesicular transporter function, c) inhibits the metabolism of the monoamine neurotransmitters by inhibiting monoamine oxidase and d) stimulates the intracellular receptor TAAR1, that induces internalization or transporter reversal of DAT increasing the flow of dopamine to the synaptic cleft and reuptake inhibition (Martin & Le, 2025). Thus, amphetamines are potent indirect dopamine agonists that can affect PKA activity by the increased dopamine levels in the synaptic cleft and the subsequent activation of dopamine receptors (Sulzer et al., 2005). Behaviorally, amphetamine can induce a variety of dose-dependent behavioral changes, with low doses leading to hyperactivity, reduced sleep and increased novelty-seeking, while higher doses can lead to stereotypic behavior, dyskinesia, seizures and psychosis-like symptoms that are commonly linked to schizophrenia (Deng et al., 2025; Mullen et al., 2025).

1.4.2 PLPPR3-dependent regulation of intracellular signaling pathways

Besides recent advances in understanding upstream regulation of PLPPR3 by signaling pathways such as the PKA pathway discussed above, PLPPR3 has emerged also as a potential regulator of intracellular signaling pathways that control neuronal function, synaptic plasticity and behavior. Specifically, PLPPR3 has been linked to the PI3K-Akt pathway via its regulatory interactions with Phosphatase and tensin homolog (PTEN) (Brosig et al., 2019; Fuchs et al.,

2020, 2022). PTEN is a tumor suppressor lipid phosphatase that dephosphorylates phosphatidylinositol (3,4,5)-trisphosphate (PIP3), thus inhibiting the PI3K-Akt pathway. PLPPR3 has been seen to regulate PTEN's activity by affecting its localization and interaction with PIP3 and other molecules in order to facilitate axon membrane branching b(Brosig et al., 2019). This inhibitory PTEN regulation indirectly leads to activation of the PI3K-Akt pathway.

The PI3K-Akt pathway is a central signaling axis that responds to extracellular growth factors and neuromodulators (Zhang et al., 2024). Activation of PI3K leads to phosphorylation of Akt, which then phosphorylates and inhibits Glycogen Synthase Kinase 3 (GSK3) (Zhao et al., 2024). GSK3 is known to negatively regulate synaptic plasticity (Bradley et al., 2012), so its inhibition by Akt is generally associated with enhanced learning, memory formation and mood stabilization (Beaulieu et al., 2009). In neurons, this pathway promotes dendritic spine maturation, LTP induction and neuroprotection (Jaworski et al., 2005; Yoshii & Constantine-Paton, 2007).Phosphorylated Akt also activates the mechanistic Target of Rapamycin (mTOR) downstream pathway which regulates local protein synthesis at the synapses (Cammalleri et al., 2003). This translational control mechanism is critical for late-phase LTP, dendritic spine remodeling and the strengthening of long-term memories (Sutton & Schuman, 2006). Phosphorylation of the S6 ribosomal protein subunit reflects mTORC1 activity and is tightly regulated by both upstream kinases like Akt and by synaptic activity (Pirbhoy et al., 2017).

These pathways are also deeply implicated in psychiatric and neurodevelopmental disorders, including autism spectrum disorder (ASD), schizophrenia, depression and bipolar disorder (Matsuda et al., 2019). Dysregulation of Akt-GSK3 signaling has been observed in schizophrenia (Emamian, 2012), while aberrant mTOR signaling is associated with ASD and intellectual disability (Thomas et al., 2023).

Apart from the importance of PLPPR3 in generating axonal filopodia through the PTEN-PI3K pathway, PLPPR3 has been seen to interact with RDX for proper thalamocortical axon guidance (Cheng et al., 2016). PLPPR3 is present in the growth cones of thalamocortical projections and PLPPR3 depletion leads to misrouting of these projections. ATX inhibition and subsequent decreased levels of LPA results in similar phenotypes like PLPPR3 KO, adding ATX derived LPA as an important factor for correct guidance together with PLPPR3. RDX is a member of the ERM protein family and a critical crosslinker between the F-actin cytoskeleton and the plasma membrane as it binds to F-actin and plasma membrane phospholipids. Interestingly, LPA induces phosphorylation of T564-RDX via RhoA-dependent ROCK kinases and increases the interaction of RDX with PLPPR3, while PLPPR3 KO results in decreased LPA-induced phosphorylation of RDX and translocation from the center to the growth cone membrane in cultured neurons. These data have suggested the presence of a ATX/LPA/PLPPR3/phosphoRDX axis that controls guidance of thalamocortical axons in vivo (Fuchs et al., 2022; Cheng et al., 2016). Notably, PLPPR3 in this context acts as a LPA sensor that enables LPA-induced axonal responses. However, whether this sensor activity depends on direct or indirect interactions with LPA is not established. Independent analyses have

suggested direct interactions of PLPPR3 with LPA (Polyzou, 2024) that may contribute to the interaction of PLPPR3 with LPA signaling and related functions.

1.5. Objectives of This Study

Despite the apparent dominant role of PLPPRs in CNS disorders, the behavioral phenotypes of PLPPR3 KO mice have not been thoroughly explored so far. Furthermore, the relation to bioactive lipids such as LPA and dopaminergic activity-regulated PKA are not known. Amphetamine strongly activates dopaminergic circuits and engages signaling cascades such as cAMP-PKA, ERK-CREB and Akt-mTOR, all of which intersect with known or predicted targets of PLPPR3. Yet, PLPPR3's influence on behavioral responses to amphetamine, or its role in shaping regional and compartment specific molecular signaling profiles, remains unknown.

This thesis addresses these fundamental questions by combining behavioral phenotyping with molecular analysis in PLPPR3 knockout and wild-type mice. The study aims to define both the baseline behavioral phenotype associated with PLPPR3 loss and the dynamic changes in behavior and signaling that occur following acute pharmacological challenge.

Specifically, the aims of this thesis are to:

1. Characterize the baseline behavioral phenotype of PLPPR3 knockout mice, using a validated battery of assays including the Open Field Test, Elevated Plus Maze, Marble Burying Test and an acute stress exposure paradigm. These tests assess locomotor activity, anxiety-like behavior, compulsive behavior and stress responses to determine whether PLPPR3 deletion influences baseline emotional and behavioral states.
2. Explore how amphetamine exposure affects behavior and signaling in wild-type versus PLPPR3 knockout mice. This includes assessment of amphetamine-induced locomotor activation, with an emphasis on the PKA signaling axis, including PLPPR3 phosphorylation at S351 and its downstream effects on synaptic scaffolding and neurotransmitter release.
3. Analyze region- and compartment-specific molecular signatures in synaptic fractions isolated from PFC and hippocampus under both basal and amphetamine-stimulated conditions. Molecular markers, such as phospho-Akt, phospho-GSK3, phospho-ERK, phospho-CREB and phospho-S6 will be examined to determine how PLPPR3 deletion alters the intracellular signaling landscape of these critical regions involved in emotion, cognition and dopaminergic function.
4. Search for possible interaction of our behavioral PLPPR3 knockout model with acute ATX inhibition and subsequent LPA level alterations.

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2. Materials and Methods

Table 1: List of antibodies used in this thesis

Antibody	Dilution (WB)	Catalog Number	Source
PLPPR3	1:1000	custom made	Eickholt lab (Brosig et al., 2019)
Phospho-PLPPR3 (S351)	1:1000	custom made	Eickholt lab (Kroon et al., 2024)
Akt	1:5000	CST9272	Cell Signaling
Phospho-Akt (S473)	1:5000	CST4060	Cell Signaling
p44/42 MAPK (Erk1/2)	1:5000	CST4695	Cell Signaling
Phospho-p44/42 MAPK (Erk1/2) (T202/Y204)	1:5000	CST9101	Cell Signaling
GAPDH	1:10000	CB1001	Sigma-Aldrich
ERM (Ezrin/Radixin/Moesin)	1/2000	3142T	Cell Signaling
Phospho-Ezrin(Thr567)/Radixin (T564)/Moesin (T558)	1:1000	3726T	Cell Signaling
PSD-95 clone K28/43	1:2000	75-0208	Antibodies Incorporated
Synaptophysin	1:2000	S5768	Sigma-Aldrich
a-Tubulin	1:10000	T6199	Sigma-Aldrich
S6 Ribosomal Protein	1:5000	2217S	Cell Signaling
Phospho-S6 (S235/236)	1:2500	4856S	Cell Signaling
Phospho-CREB (S133)	1:5000	ab32096	Abcam
Phospho-GSK-3β (S9)	1:2500	cst9323	Cell Signaling
GluA1 pS845 (clone D10G5)	1:1000	8084	Cell Signaling
BASP1	1:1000	Cell Signalling (test sample)	Eickholt lab (Kroon et al., 2024)
Anti-mouse peroxidase labeled (H&L)	1:10000	Vector	PI-2000
Anti-rabbit peroxidase labeled (H&L)	1:5000	Vector	PI-1000

Table 2: List of solutions, buffers and kits used in this thesis.

Reagent/Kit	Catalog Number	Source
DMSO	D2650	Sigma
BCA Protein Assay Kit	71285-3	Millipore
Clarity Western ECL Substrate	170-5061	Biorad
PCR-grade water	PCR-2585.1	Iena Biosciences

TEMED	1.10732.0100	Sigma-Aldrich
Tween 20	P1379 -1L	Sigma-Aldrich
NucleoSpin Tissue Kit	740952.50	Macherey-Nagel
DNA ladder	MWD100	Nippon Genetics
PCR Kit	KK1509	Kapa Biosystems
2-Mercaptoethanol	M6250	Sigma-Aldrich
Glycerol	BP229-1	Fisher BioReagents

Table 3: Powder reagents and laboratory chemicals used in this thesis.

Antibody	**Catalog Number**	**Source**
Agarose	A9539	Sigma-Aldrich
Tris base	T1503	Sigma-Aldrich
Glycine	1.00590	Sigma-Aldrich
Sodium fluoride	201554	Sigma-Aldrich
Sodium orthovanadate	567540	Sigma-Aldrich
B-Glycerophosphate disodium	35675	Sigma-Aldrich
Phenylmethanesulfonylfluoride	P7626	Sigma-Aldrich
Sodium molybdate	243655	Sigma-Aldrich
Boric Acid	31146	Honeywell
Bromophenol blue	161-0404	Bio-Rad
EDTA	ED2P	Sigma-Aldrich

2.1. Animals

Male C57Bl/6NCrl wild-type (WT) and PLPPR3 knockout (KO) mice (C57 Bl/6NCrl Plppr3$^{-/-}$) were obtained from the Animal Facility at the University of Ioannina (license No. "EL33-BIObr01"). The PLPPR3 KO mice were established by Brosig et al. (Brosig et al., 2019). Mice were housed in plastic cages (47.5 cm length × 20.5 cm height × 27 cm width), with a maximum of six animals per cage. They were maintained in a climate-controlled environment with a 12-hour light/dark cycle (lights on at 7:00 AM) and provided ad libitum access to food and water. All experiments were performed during the light phase of the cycle. The protocols were approved by the Institutional Animal Facility Committee of the University of Ioannina and complied with the European Union Directive 2010/63/EU on the protection of animals used for scientific purposes.

2.2. Behavioral Tests: Open Field, Elevated Plus Maze, Marble Burying Test

Behavioral Procedures

All behavioral experiments were conducted following the approval of our protocols by the local authorities (protocol number: 2332, date: 23/02/2024). To ensure reproducibility and minimize handling-related stress, all animals underwent a systematic pre-experimental habituation. Mice were gently handled once daily for a period of seven consecutive days prior to the onset of behavioral assessment (Ghosal et al., 2015). During handling, animals were lifted, gently restrained, and allowed to explore the experimenter's hands for a few minutes to reduce novelty- and handling-induced stress during testing (Hurst & West, 2010; Sensini et al., 2020). On the day preceding behavioral experiments, mice were transferred to the behavioral testing room and left undisturbed to acclimatize for 24 hours to the new environmental conditions, including light intensity, ambient noise, and odor cues (Prut & Belzung, 2003). All behavioral tests were performed during the light phase to reduce circadian-related variability.

Open Field Test (OFT)

The open field test (OFT) was used to assess general locomotor activity and anxiety-like behavior (Seibenhener & Wooten, 2015). The apparatus consisted of a transparent Plexiglas box (40×40×40 cm), open from above, and equipped with 16 horizontal and vertical photocells linked to an electrical recording unit (ENV515, Activity Monitor, version 5, Med Associates). Mouse movement was recorded when the animals interrupted the light beams, and various mobility parameters were stored digitally, including horizontal mobility (distance traveled, duration, frequency of ambulatory events) and vertical mobility (duration and frequency of rearing). The recording period was set at 60 minutes. After each test, the arena was cleaned with water and 70% ethanol to remove any odor traces.

Elevated Plus Maze (EPM)

The Elevated Plus Maze (EPM) test was used to evaluate anxiety-like behavior in mice (Walf & Frye, 2007). The apparatus consisted of two open arms (30×5 cm) and two closed arms (30×5×15 cm) extending from a central platform (5×5 cm), elevated 60 cm above the floor. To enhance the contrast between the open and closed arms, closed arms were covered with removable lids, as previously described in studies using modified EPM designs (Doukkali et al., 2016). Mice were placed individually on the central platform facing an open arm and allowed to freely explore the maze for 5 minutes. Time spent in open and closed arms, as well as the number of entries into each arm, were recorded using a camera. An entry was defined by the movement of all four paws of the animal to a different compartment. The apparatus was cleaned with 70% ethanol between trials to eliminate odor cues. Anxiety-like behavior was inferred based on the time spent in open arms and the number of open arm entries, with decreased time and entries indicating increased anxiety. All behaviors were scored using Behaview (https://www.pmbogusz.net/?a=behaview).

Marble Burying Test (MBT)

The marble burying test (MBT) was used to assess anxiety-like and compulsive behaviors in mice (Himanshu et al., 2020). The test was conducted in a standard cage (30×20×20 cm) filled with 5 cm of fresh bedding material. A total of 20 glass marbles (15 mm diameter) were evenly spaced (4×5 grid) on the bedding surface. Each mouse was placed individually in the cage and allowed to explore freely for 30 minutes. At the end of the test, the number of buried marbles (defined as at least two-thirds covered with bedding) was recorded. The apparatus was cleaned with 70% ethanol between trials to remove any scent cues. Increased marble burying activity was interpreted as increased anxiety or compulsive-like behavior, while reduced activity suggested lower levels of such behaviors.

2.3. Acute stress

To induce acute stress, mice were subjected to a restraint stress protocol (Schmidt, 2024). Animals were restrained for 30 minutes in a modified 50 ml, clear polypropylene tube (diameter 3 cm, 12 cm long) with multiple air holes for ventilation. The tubes were sized appropriately to restrict movement without causing harm or discomfort to the mice. Following the restraint period, mice were immediately tested in the OFT or EPM. This method was employed to evaluate the physiological and behavioral responses to acute stress in WT and PLPPR3 KO mice.

2.4. Drug administration

Amphetamine experiment: Amphetamine (4 mg/kg, i.p.) was administered to mice (Yates et al., 2007), while control animals received saline injections. Ten minutes post-injection, mice were placed in the open field arena for behavioral assessment. For investigation of amphetamine-induced changes in signaling, animals were injected again with saline or amphetamine after a 3-day wash-off period (in an inverse order), euthanized and brain tissue samples were isolated and snap-frozen.

Autotaxin inhibitor: To investigate the effects of a known autotaxin inhibitor, PF-8380 (Selleckchem, Cat. No. S8218), mice were administered the compound at a dose of 30 mg/kg (i.p.) (Gierse et al., 2010). PF-8380 was diluted in DMSO at a stock solution. The working solution consisted of 5% DMSO, 5% Cremophor, and 90% saline (NaCl). Behavioral testing in the elevated plus maze (EPM) was conducted three hours post-injection in accordance with Thalman et al. (Thalman et al., 2018). Immediately after the EPM test, mice were euthanized,

and brain tissue samples were isolated and snap-frozen for the evaluation of possible signaling changes from autotaxin inhibition

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2.5. Tissue Collection

For protein isolation, WT and PLPPR3 KO male C57BL/6 mice were sacrificed by decapitation. Hippocampus, prefrontal cortex, striatum and amygdala from 3 months old mice were dissected and snap-frozen for subsequent molecular analyses.

For DNA extraction, tails (1-2 mm) from WT and PLPPR3 KO C57BL/6 mice were dissected during the weaning period (PND21).

2.6. Genotyping protocol (Isolation of DNA, PCR, Electrophoresis)

DNA isolation: DNA was isolated using the NucleoSpin Tissue Kit (Macherey-Nagel) following the manufacturer's protocol adjusted for tail samples. Briefly, tissue samples were homogenized in a lysis buffer containing proteinase K and incubated at 56°C until completely lysed. After lysis, DNA was bound to the silica membrane within the NucleoSpin columns by applying the lysate and centrifuging. The columns were washed with the provided buffers to remove contaminants, and the DNA was eluted in a low-salt buffer. The quality and concentration of the extracted DNA were assessed using a spectrophotometer and stored at -20°C until further use.

PCR: For the DNA sample genotyping, Polymerase Chain Reaction (PCR) was performed using the KAPA BIOSYSTEMS HotStart PCR kit (Cat. No. KK1509). The PCR assay was performed using the BIO-RAD C1000 Dx Thermal Cycler with the CFX DX ORM Real-Time PCR System. Reaction conditions are detailed in Tables 4 and 5. Amplification was carried out using a forward primer (5') and two alternative reverse primers (3'), enabling distinction between wildtype (WT), knockout (KO), and heterozygous genotypes.

- The **first primer pair** (forward + reverse 1) amplified an 883 bp fragment in WT animals and a 148 bp fragment in KO animals (Brosig et al., 2019). In heterozygous animals, both fragments were amplified, typically with reduced intensity due to monoallelic presence (Figure 2.1 A).
- A **second reverse primer** targeting a different region of the gene, when combined with the same forward primer, amplified a 194 bp product exclusively in WT samples. No amplification was observed in KO samples, serving as an internal control for genotyping specificity (Figure 2.1 B).

Table 4. Reagent concentrations and quantities per sample

Reagent	Initial Concentration	Final Concentration	Volume per Reaction (µL)
PCR-grade water			12.65
KAPA Taq HotStart Buffer	5x	1x	5
$MgCl_2$	25 mM	1.5 mM	1.5
dNTP Mixture	10 mM	0.2 mM	0.5
Forward Primer (5')	10 µM		1
Reverse Primer (3')	10 µM	1µM	1
DMSO		5%	1.25
HotStart Taq	5 U/µl	0.5 U	0.1
Genomic DNA (gDNA)			2
Final Volume			25

Table 5. PCR Cycle Program

Step	Temperature	Duration	Cycle Count
Lid Heating	95°C		1
Initial Denaturation	95°C	3 minutes	35 cycles
Denaturation	95°C	30 seconds	
Annealing	50°C	30 seconds	
Extension	72°C	1 minute, 10 seconds	
Final Extension	72°C	10 minutes	1
Hold	8°C	∞	1

Primers used for genotyping:
Forward Primer (5'): 5’ CAG GGA CCT CAC CAT GGA AAC G 3’
First reverse Primer (3'): 5’ TTG CAA CTC CTA CTC GAC CTG 3’
Second reverse Primer (3'): 5’ GTG CCT TCT GTG CGC CTT G 3’

Electrophoresis Procedure: PCR products were subjected to electrophoresis on a 2% agarose gel. For the gel preparation, 2 g of agarose were weighed and dissolved in 100 mL of 1X Tris-Borate-EDTA (TBE) buffer (Tris-base 0.09 M, Boric Acid 0.09 M and EDTA 0.002 M). Finally, 10 µL of RedSafe dye were added (Biochem, catalog number 41003) for DNA band visualization. RedSafe binds to DNA, allowing band visualization. A 3 µL DNA ladder (MWD100) and 20 µL of DNA samples were loaded into each well. The electrophoresis was conducted using the Mupid® One Electrophoresis System Complete Apparatus.

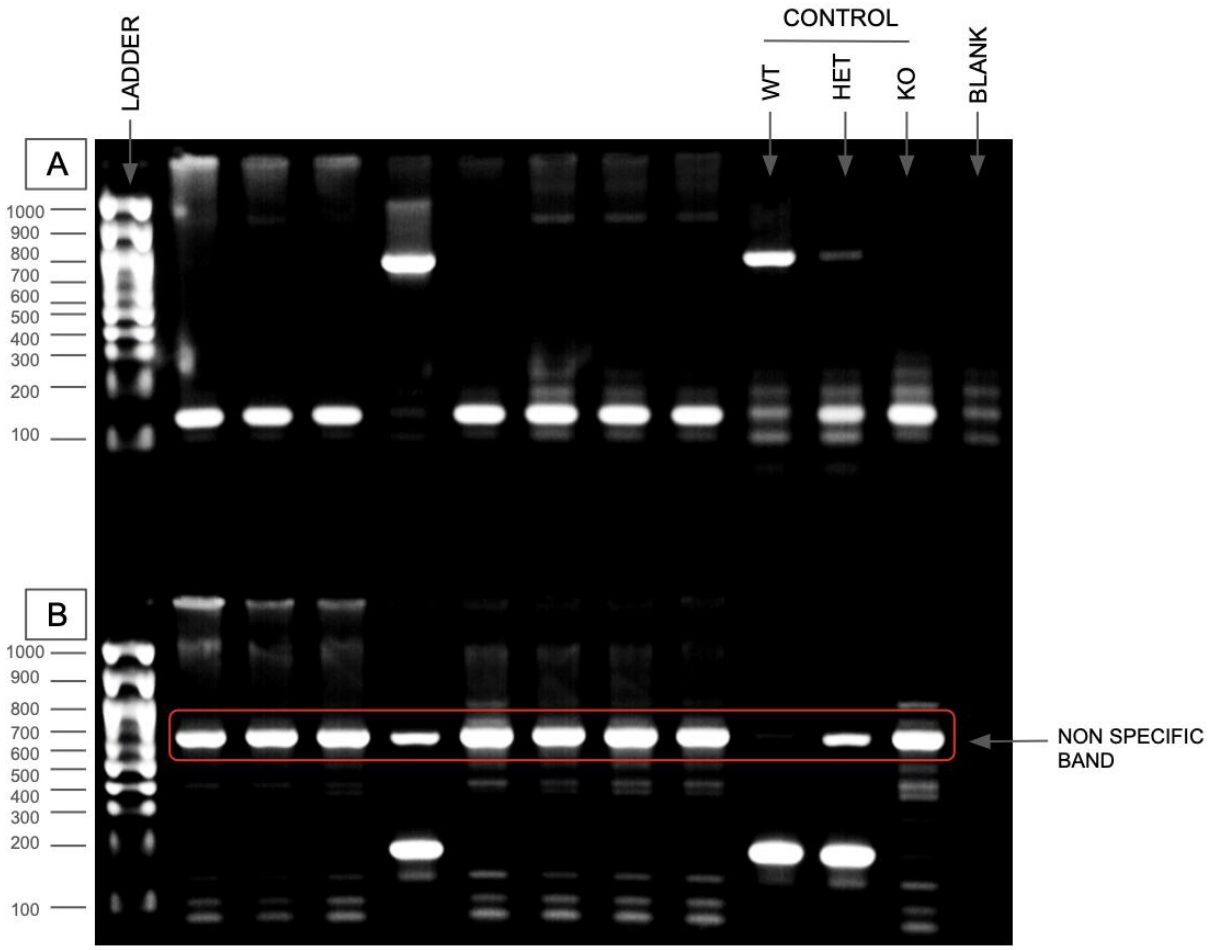


Figure 2.1 Genotyping Results: (A) Gel image showing WT (883 bp), KO (148 bp), and heterozygous (both bands) patterns using the first reverse primer. (B) Control PCR with second reverse primer, showing 194 bp band only in WT samples, absent in KO. A non-specific band was present (~650bp). Ladder band lapels in bp.

2.7. Protein Isolation and Western Blot assay

Protein Isolation: Tissues were lysed and homogenized in RIPA buffer supplemented with phosphatase inhibitors (2 mM sodium fluoride, 2 mM sodium orthovanadate, 2 mM β-glycerophosphate, 2 mM sodium molybdate), 0.2 mM PMSF, and 0.4% Protease Inhibitor Cocktail (Calbiochem, #539134). After protein solubilization and centrifugation at 15,000 rpm for 20 minutes at 4°C, the supernatants were aliquoted and stored at -80°C. Protein concentrations were determined using the BCA Assay Kit (Pierce II, USA).

Western Blot assay:

Sample preparation: Protein samples were mixed with Laemmli Buffer/SDS-PAGE Loading Buffer (Tris-HCl pH:6.8, SDS 6%, Glycerol 48%, 2-Mercaptoethanol 9%, Bromophenol blue

0.03%) in a final concentration of 2 μg/μl. Then protein samples were denatured with heat at 96°C for 4 minutes and could be stored at -20°C.

SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis): 25 μg of protein sample, a protein ladder and appropriate internal control samples were loaded in 8 or 10% SDS-PAGE gels. Electrophoresis was done at a constant voltage (e.g., 80-120V) until the dye front reached the bottom of the gel. Running time varies depending on the gel percentage and voltage (typically 1-2 hours).

Protein transfer: Protein samples were then transferred to a nitrocellulose membrane at 400 mA for 2 hours at 4°C. After transfer, membranes were briefly stained with Ponceau S to visualize total protein transfer and confirm even loading. Membranes were then rinsed with water and TBST to remove Ponceau S stain.

Blocking: Membranes were placed in a clean tray with sufficient blocking buffer (5% milk in 0.1% TBS-T (TBS + 0.1% Tween-20) and incubated on a rocker or shaker at room temperature for 1 hour

Primary Antibody Incubation: Blocking buffer was then removed from the membrane and diluted primary antibody solution was added. Membranes were incubated on a rocker or shaker at 4°C overnight.

Washes: Primary antibody solution was collected, and membranes were washed three times for 10 minutes each with fresh wash buffer TBS-T, on a rocker or shaker at room temperature.

Secondary Antibody Incubation: The diluted secondary antibody solution was added to the membrane and then incubated on a rocker or shaker at room temperature for 1 hour.

Washes: Secondary antibody solution was poured off and membranes were washed three times for 10 minutes each with fresh wash buffer TBS-T, on a rocker or shaker at room temperature.

Detection: Membranes was incubated with enhanced chemiluminescence substrate (Clarity, Bio-Rad, USA) and imaged using the ChemiDocTMXRS+ imaging system (Bio-Rad, USA). Band intensities were quantified using Fiji, and statistical analysis was performed using GraphPad Prism software (version 8.0). Normalization of all densitometry values for all antibodies tested in all protein samples was performed by dividing signals with the signal of an internal control sample (i.e. WT mouse striatal protein sample) that was included in every gel.

2.8. Synaptosome Isolation

Synaptosomes were isolated from the hippocampus of adult WT and PLPPR3 KO mice using a protocol from Takasmim's lab (Sahara et al., 2014) modified by Dr. Charis Brakatselos (Laboratory of Pharmacology, University of Ioannina). Tissue (approximately 40 mg) was homogenized using a homogenate buffer containing 9% sucrose, 5 mM DTT, 2 mM EDTA, 25 mM Tris (pH 7.4), and protease and phosphatase inhibitors. After centrifugation at 1,000g for

five minutes, the nuclear fraction (P1) was separated. The supernatant (S1) underwent further centrifugation at 12,500g for fifteen minutes. The resulting microsomal and cytosolic fraction (S2) was saved. The synaptosomal membrane fraction (LP1) was isolated by centrifugation at 25,000g for twenty minutes. LP1 fractions were analyzed by Western blotting, and synaptosomal quality was assessed by the presence of postsynaptic marker PSD-95 and presynaptic marker synaptophysin.

2.9. Statistics

Statistical analyses were performed using GraphPad Prism software (version 8.0.1). Data are presented as mean ± SEM. For comparisons between two groups (WT and PLPPR3 KO), unpaired two-tailed Student's t-tests with Welch's correction were used. For experiments involving multiple parameters, such as the drug administration experiments, two-way analysis of variance (ANOVA) was performed, followed by Tukey's multiple comparisons test to assess differences between groups. Statistical significance was set at $p < 0.05$, and p-values are reported as follows: ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

3.Results

3.1. Behavioral Results

3.1.1. PLPPR3 KO mice display increased locomotion activity and exploratory behavior compared to WT in the OFT

The OFT was contacted to measure the baselines of our animal model C57Bl/6NCrl PLPPR3 KO mice in comparison to WT controls (C57Bl/6NCrl WT). This test evaluates the general locomotor activity and anxiety-like behavior through thigmotaxis. Additionally, it allows for assessment of habituation over time as there was no habituation period for the animals in the OFT before the test, thus providing insight into novelty reactivity and adaptation. Generally, the first half (0-30 minutes) is considered as the period of reaction to novelty and the second half (30-60 minutes) as the post-habituation period.

PLPPR3 KO mice traveled more distance than WT mice (Figure 3.1A) and a tendency of difference was detected in the percentage of distance traveled and total time spent in the inner-central zone, with KO mice having increased percentages in both parameters (Figure 3.2). No statistical differences were detected comparing the total rearing events (Figure 3.3). In both habituation and post-habituation halves KO mice traveled significantly more distance compared to WT (Figure 3.3). In addition, in 15-minute block comparison KO mice had traveled higher distances compared to WT mice with significant differences in all four 15-minute blocks (Figure 3.4A). Cumulatively, the significantly increased distance covered for KO mice is again evident (Figure 3.4B).

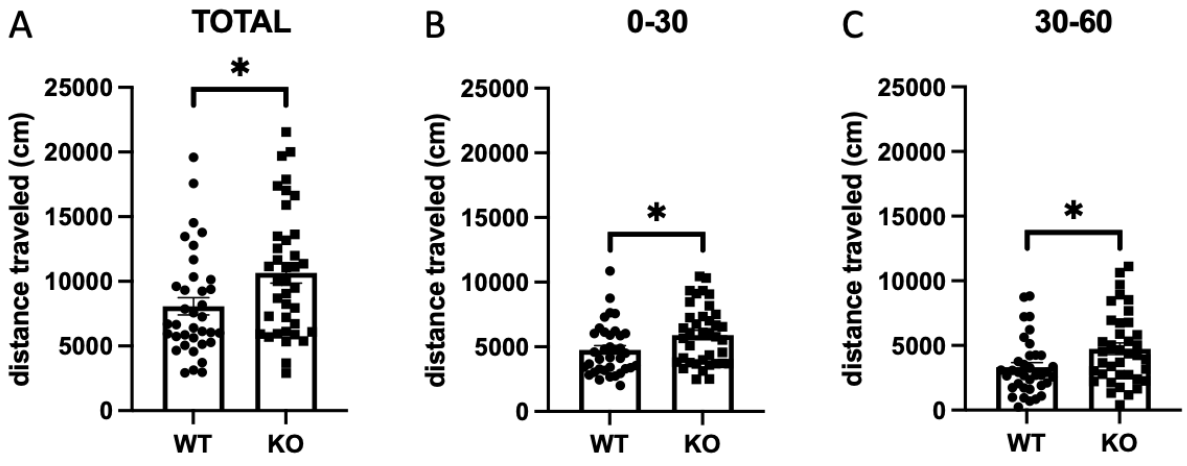


Figure 3.1 Open Field distance traveled. A. Total distance traveled (cm) in the OFT by WT and PLPPR3 KO mice. KO mice exhibited significantly increased locomotor activity compared to WT mice (p=0.0152). B. Distance traveled in the habituation period (0-30 minutes). KO mice exhibited significantly increased locomotor activity compared to WT mice (p=0.0226) C. Distance traveled in the post-habituation period (30-60 minutes). KO mice exhibited significantly increased locomotor activity compared to WT mice (p=0.0152). WT: n=36, KO: n=38.

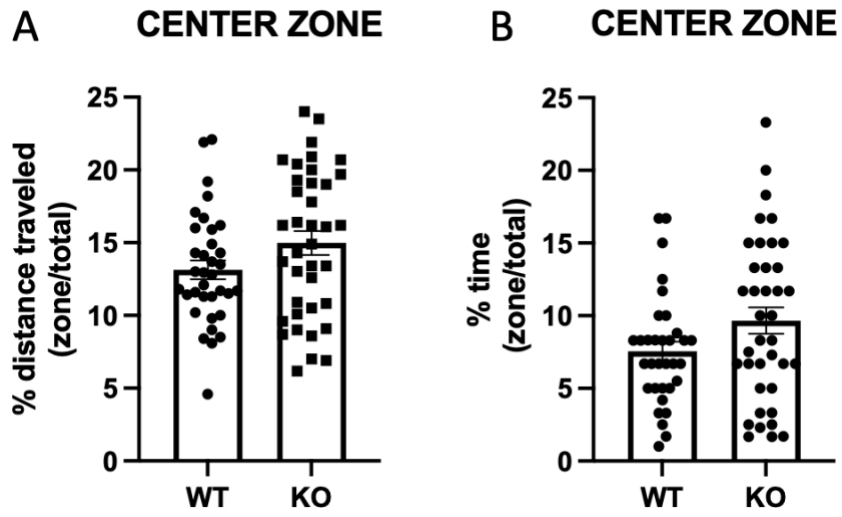


Figure 3.2 A. Percentage of total distance traveled, and B. total time spent in the inner zone of the OFT. KO mice showed a tendency towards increased exploration of the inner zone (A. p=0.1; B. p=0.0671), but differences did not reach statistical significance. WT: n=36, KO: n=38.

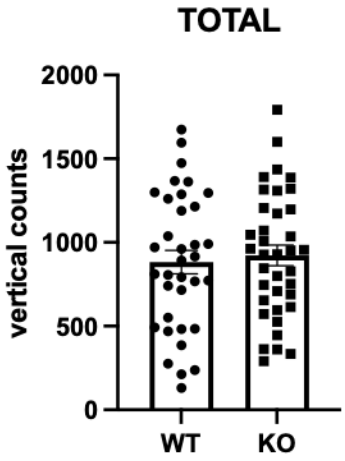


Figure 3.3 Total vertical counts. Number of vertical counts (rearing events) recorded in the OFT. No significant differences were observed between genotypes. WT: n=36, KO: n=38.

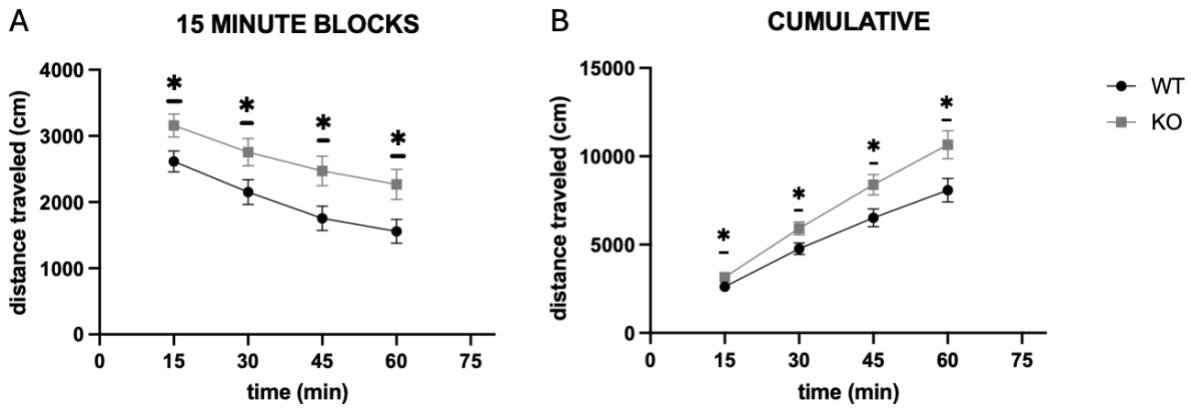


Figure 3.4 OFT results per 15-minute blocks. A. Distance traveled (cm) in the OFT separated into 15-minute time blocks. KO mice exhibited significantly increased locomotion compared to WT mice across all time intervals with p=0.0226 first block (0-15 minutes), p=0.0343 second block (15-30 minutes), 0.0161 third block (30-45 minutes), 0.0172 fourth block (45-60 minutes). B. Distance traveled (cm) in the OFT cumulatively per 15-minute time blocks. KO mice exhibited significantly increased locomotion compared to WT mice across all time intervals with p=0.0226 first block (0-15 minutes), 0.0226 second block (0-30 minutes), 0.0171 third block (0-45 minutes), 0.0152 fourth block (0-60 minutes). WT: n=36, KO: n=38.

3.1.2. PLPPR3 KO mice exhibit a reduced anxiety-like behavior compared to WT mice in the EPM

The OFT results suggested a statistically significant increased exploratory behavior for PLPPR3 KO mice and furthermore, a trend towards an anxiolytic phenotype, depicted via the increased percentages spent in the inner zone (Figure 3.2). Therefore, we used the EPM test to formally evaluate anxiety-like behavior. This task relies on the natural aversion of rodents to open and elevated areas. Time spent in open arms versus closed arms provides an index of anxiety-like behavior, while total arm entries reflect general locomotion and exploratory drive.

WT mice spent significantly more time in the closed arms than KO mice (Figure 3.5A), with the KO spending significantly increased time in the open arms (Figure 3.5B). No differences were observed in total entries through the apparatus (Figure 3.6C), with a significantly higher number of entries in the open arms for the KO mice compared to WT mice (Figure 3.6B).

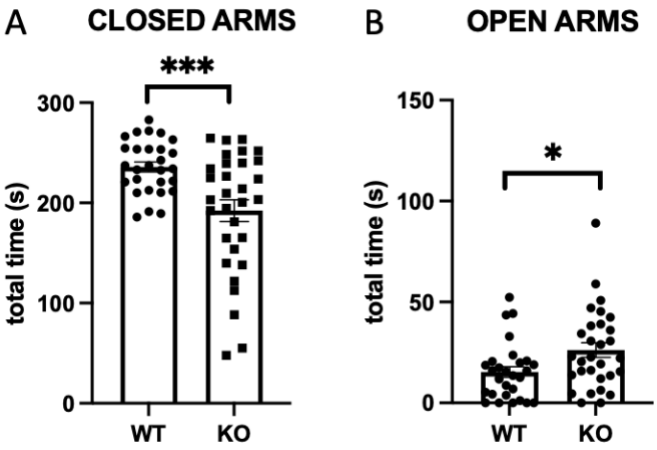


Figure 3.5 EPM total time in closed and open arms. Time spent in the closed and open arms of the EPM by WT and KO mice. A. KO mice spent significantly less time in the closed arms (p=0.0007) and B. more time in the open arms (p=0.0189) compared to WT mice. WT: n=28, KO: n=31.

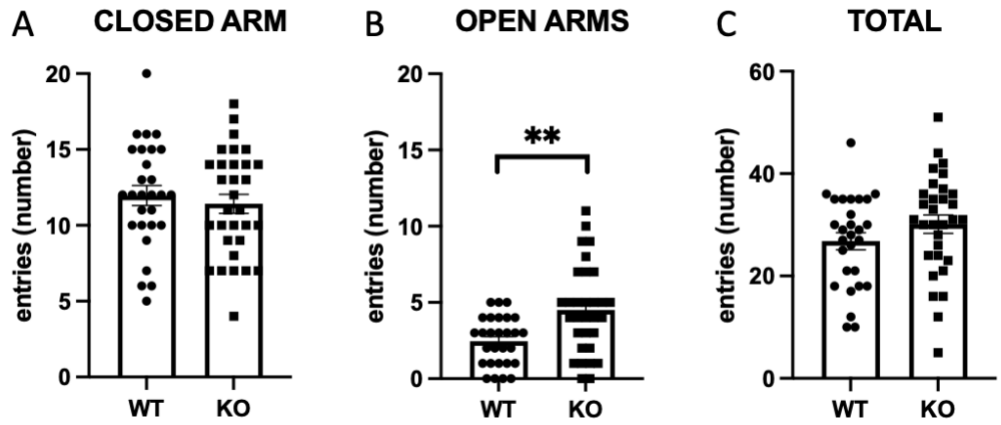


Figure 3.6 Number of entries into the arms of the EPM. A. No differences in open arm entries. B. KO mice made significantly more entries into the open arms compared to WT mice (p=0.0014). C. Total entries across arms were not significantly different (p=0.1814). WT: n=28, KO: n=31.

3.1.3. PLPPR3 KO mice display reduced anxiety- and compulsive-like behavior in the MBT

The MBT was employed in continuation of the EPM results, as an additional assessment of anxiety-related behavior. This test measures the innate tendency of mice to bury unfamiliar objects, e.g. marbles. Higher buried marble counts are often interpreted as elevated anxiety or compulsive behavior (de Brouwer et al., 2019).

During the MBT, KO mice buried significantly fewer marbles than those buried from WT mice (Figure 3.7), suggesting altered anxiety or compulsive-like behavior.

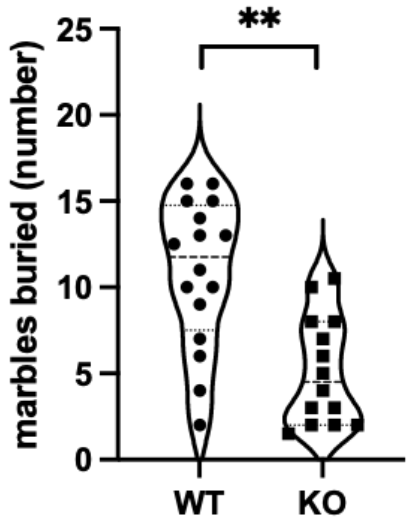


Figure 3.7 Marbles buried in MBT. Number of marbles buried in the Marble Burying Test (MBT) by WT and PLPPR3 KO mice. KO mice buried significantly fewer marbles than WT mice (p=0.0003). WT: n=16, KO: n=14.

Conclusively, the results of all three behavioral tests performed, namely OFT, EPM and MBT, converge towards a specific anxiolytic-like behavioral phenotype of PLPPR3 KO mice compared to WT mice.

3.1.4. Acute restraint stress alters locomotor and exploratory behavior of PLPPR3 WT and KO mice in a genotype-independent manner

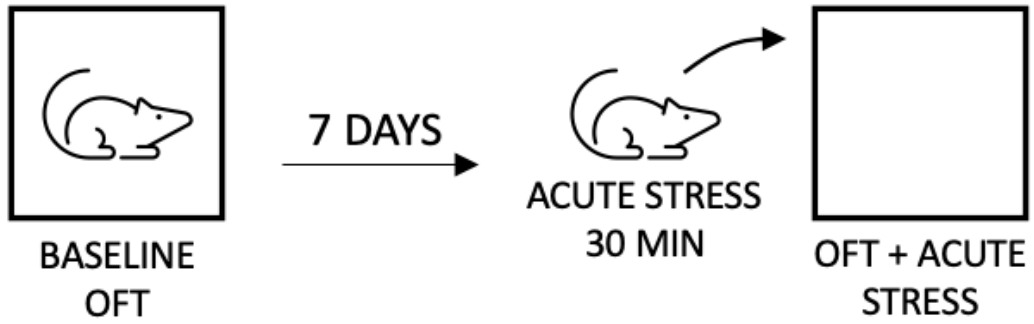


Figure 3.8 Experimental design of the acute stress experiment and open field evaluation. WT and PLPPR3 KO mice first evaluated in the OFT for baseline results. One week after the baseline OFT mice were acutely stressed through a 30-minute enclosure in 50 ml Falcon tubes. Directly after acute stress mice were evaluated in the OFT.

This experiment investigated the effect of acute restraint stress followed by OFT, using a repeated-measures design, where the same animals served as both baseline and stress-

exposed. The goal was to determine whether KO mice, which were characterized as less prone to anxiety-like behavior, can show resilience to an acute restraint stress test. The experiment started with a baseline OFT test as data for baseline parameters were needed as our groups were too small to have control and stressed groups simultaneously. One week later, to avoid any habituation effects, the acute restraint stress was conducted in all animals, with an OFT following it (Figure 3.8).

The results demonstrate that acute stress significantly impacts distance traveled, with individual differences playing a major role, while genotype and its interaction with stressor have minimal influence (Figure 3.9B). Even though data on vertical activity are inconclusive, these results highlight that acute stress appears to have minimal impact (Figure 3.9A). Finally, while the percentage of distance traveled in the zone is not influenced by stressor or genotype (Figure 3.9C), the interaction between these factors plays a significant role in determining the percentage of time spent in the zone, with individual differences affecting only the KO mice (Figure 3.9D).

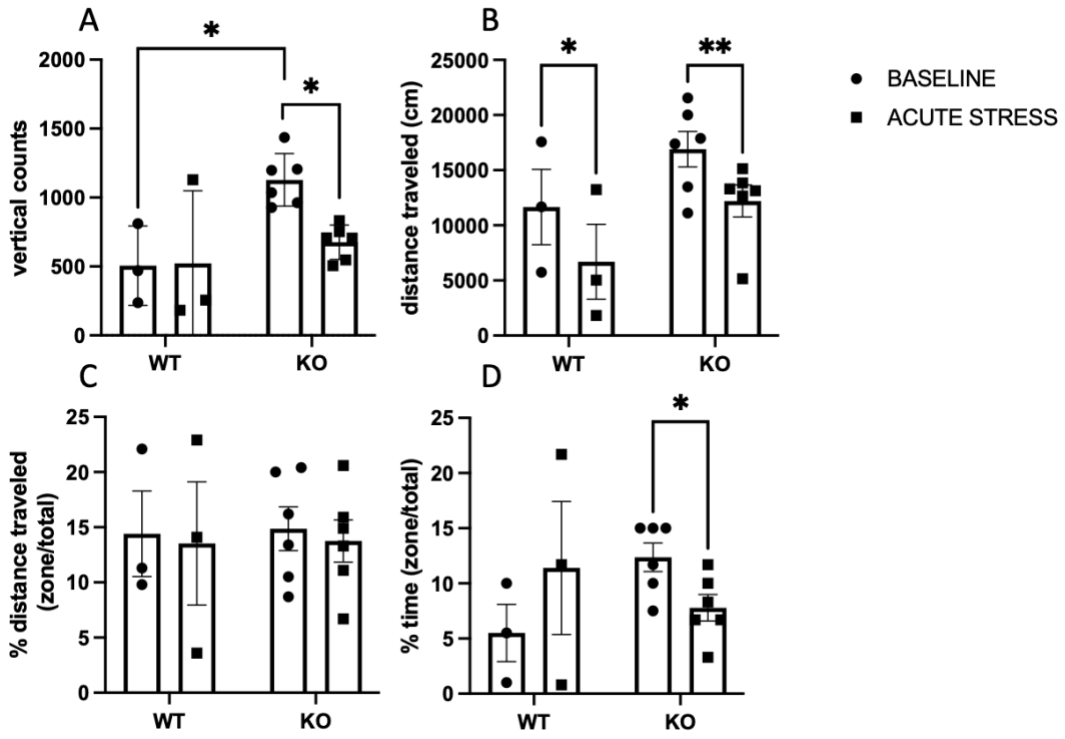


Figure 3.9 OFT alterations from baseline to acute stress. Effects of acute restraint stress on locomotor activity in the Open Field Test (OFT). A. Vertical counts (rearing events). Statistical difference between baseline WT and KO mice (p=0.010) and baseline with acute stress KO mice (p0.0092). B. Total distance traveled. Statistical difference between baseline and acute stress (WT, p=0.0290 and KO, p=0.0069). C. Percentage of distance traveled. D. Percentage of time spent in the inner zone. Statistical difference between baseline and acute stress in PLPPR3 KO mice (p=0.0067). Statistical results concerning each genotype are indicated as individual mice differences and not a genotype related difference. WT: n=3, KO: n=6.

3.1.5. Acute stress increases anxiety-like behavior of both PLPPR3 WT and KO mice

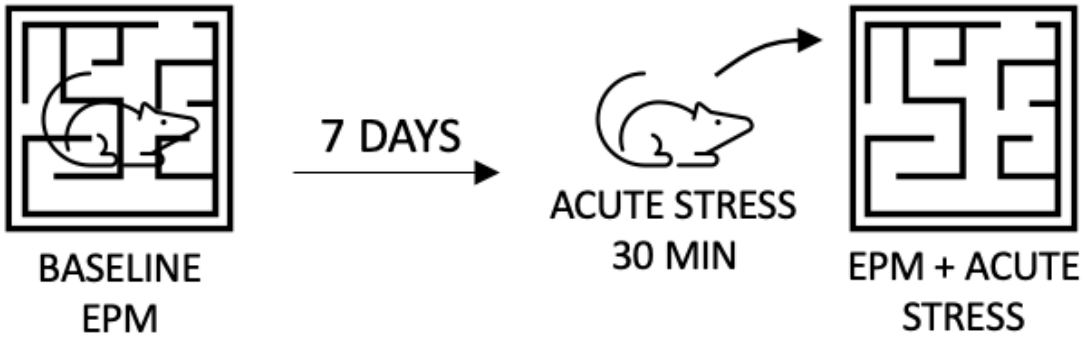


Figure 3.10 Experimental design of the acute stress experiment and the EPM evaluation. WT and PLPPR3 KO mice are first evaluated in the EPM for baseline results. One week after the baseline EPM, mice were acutely stressed through a 30-minute enclosure in 50 ml Falcon tubes. Directly after acute stress mice were evaluated in the EPM. Scheme generated with PowerPoint.

To further evaluate the impact of acute stress, another group of animals were tested in the EPM following restraint. This approach allowed for assessment of stress-induced changes in anxiety-related behavior and tested the hypothesis that PLPPR3 KO mice may differ in stress reactivity compared to WT controls. The baseline group is the same animals that were tested one week later in the EPM after the acute restraint test (Figure 3.10).

Although there was substantial variability of responses in this experiment, the analysis of behavioral outcomes in the EPM following acute restraint stress revealed significant effects of the stressor across all measures. Here, KO mice, after acute stress, had a disruption of the low anxiety-like behavioral phenotype (Figure 3.11 and 3.12). Time assessment in the closed and open arms after acute stress revealed a statistically significant effect only in KO mice (Figure 3.11). It should be noted that although WT mice displayed also a tendency towards increased time spent in closed arms and decreased time in the open arms (Figure 3.11), the results were not statistically significant, likely due to the low numbers of WT mice assessed. Baseline KO mice spent statistically more time in open arms compared to baseline WT (Figure 3.11B), in line with our abovementioned data.

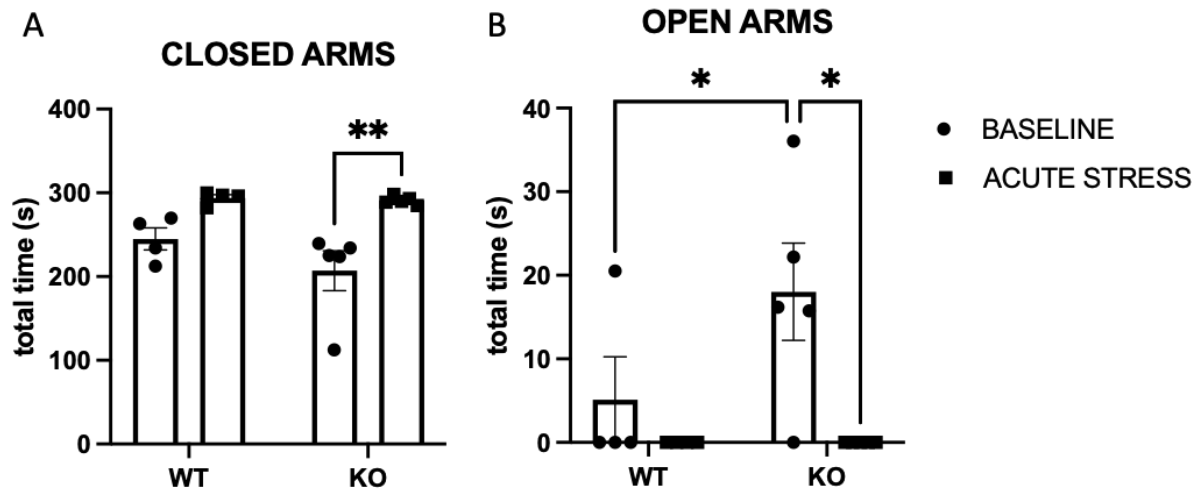


Figure 3.11 Total time in the different EPM compartments after acute stress. A. Effects of acute restraint stress on time spent in the closed arms, with significant increase for the KO mice after acute stress (p=0.0034) and a tendency for WT (p=0.0584). B. Effects of acute restraint stress on time spent in the open arms, with significant decrease for the KO mice after acute stress (p=0.0437). WT baseline mice spent less time in the open arms compared to the KO baseline (p=0.0383). Statistical results concerning each genotype are indicated as individual mice differences and not a genotype related difference. WT: n=4, KO: n=5.

On the contrary, analysis of closed arms, open arms and total entries showed a significant effect of the stressor in both WT and KO mice (Figure 3.12). It should be stressed here that a strong phenotype that was observed in both genotypes was the large decrease of total entries, suggesting reduced motility and exploration after acute stress (Figure 3.12C).

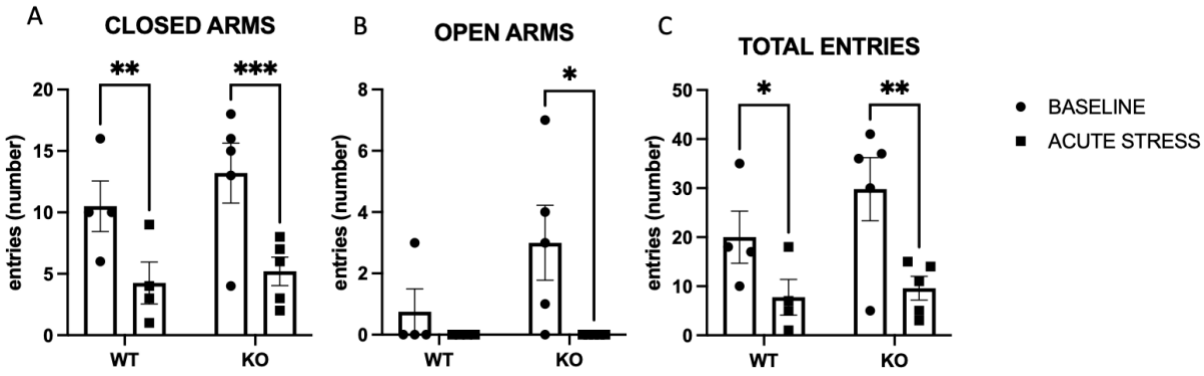


Figure 3.12 Number of entries in EPM after acute stress. A. Effects of acute restraint stress on the number of entries into the closed arms, with significant decrease for both WT and KO mice after acute stress (p=0.0043 and p=0.0005 respectively). B. Effects of acute restraint stress on the number of entries into the open arms, with significant decrease for KO mice after acute stress (p=0.0437) C. Stress significantly decreased total entries (p=0.0007), with no significant effects of genotype. Statistical results concerning each genotype are indicated as individual mice differences and not a genotype related difference. WT: n=4, KO: n=5.

3.1.6. Autotaxin inhibitor PF-8380 does not affect anxiety-like behavior

PLPPR3 has been identified as a possible atypical effector of the bioactive lipid and synaptic neuromodulator LPA (Polyzou et al., 2026). In this context, we sought to investigate whether the low anxiety phenotype of PLPPR3 KO mice can be modified upon acute changes in CNS LPA levels. PF-8380 (6-(3-(piperazin-1-yl)propanoyl)benzo[d]oxazol-2(3H)-one) is a selective inhibitor of ATX, the enzyme responsible for the synthesis of LPA. PF-8380 has been shown to acutely inhibit ATX resulting in decrease of LPA concentration in CSF of mice and correct PLPPR4 KO behavioral phenotypes (Thalman et al., 2018). This test aimed to assess whether acutely blocking LPA production via PF-8380 would modulate the anxiety-like behaviors of WT mice and/or PLPPR3 KO mice.

The analysis of behavioral outcomes in the EPM following the PF-8380 experiment revealed no significant effects of the drug, genotype, or their interaction on any of the measured parameters (Figure 3.13 and 3.14).

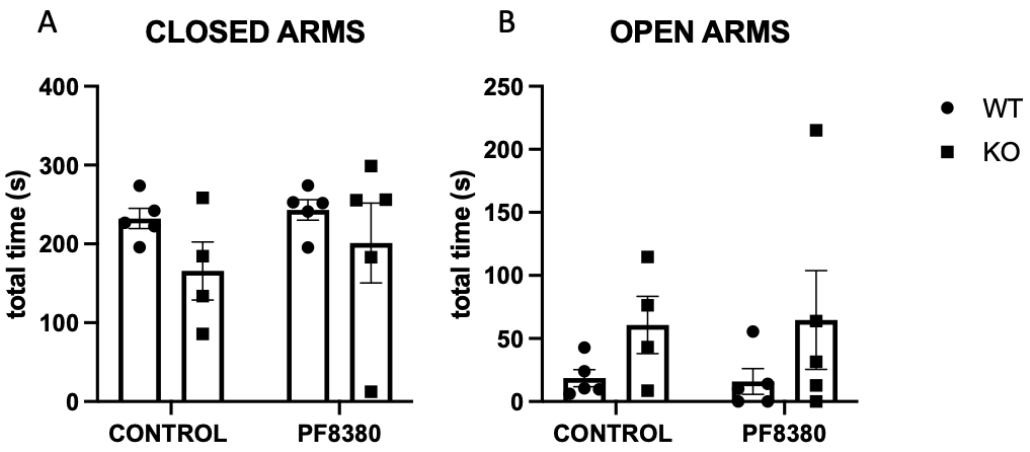


Figure 3.13 Total time in compartments of the EPM after administration of PF8380 (i.p., 30 mg/kg, 3 hours before test). A. Effects of PF-8380 administration on time spent in the closed arms. B. Effects of PF-8380 administration on time spent in the open arms. Neither the drug nor genotype had significant effects on time spent in either arm. WT control: n=5, WT PF-8380: n=5, KO control: n=4, KO PF-8380: n=5.

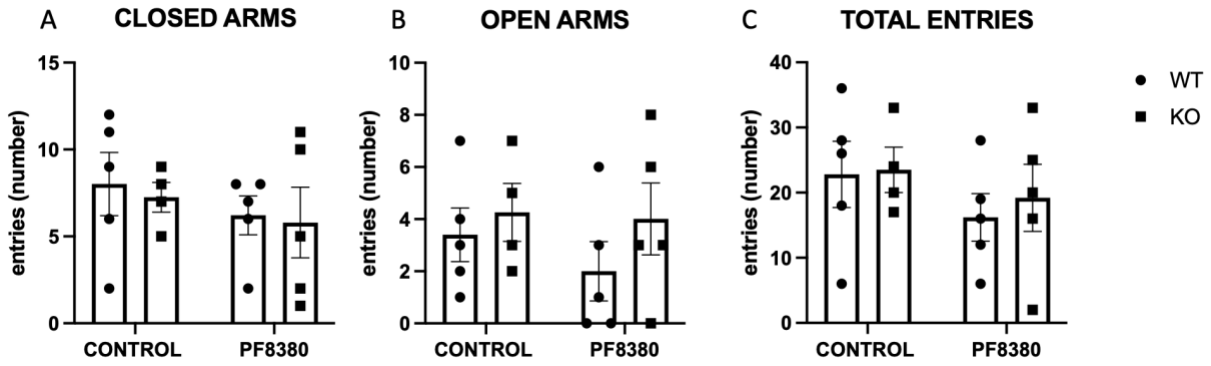


Figure 3.14 Effects of PF-8380 administration on the number of compartment entries of the EPM. A. Effects of PF-8380 administration on the number of entries in the closed arms. B. Effects of PF-8380 administration on the number of entries in the open arms. C. Effects of PF-8380 administration on total number of entries. Neither the drug nor genotype had significant effects on closed arms, open arms or total entries. WT control: n=5, WT PF-8380: n=5, KO control: n=4, KO PF-8380: n=5.

3.1.7. Amphetamine administration increases locomotion independently of genotype

The OFT was used to assess the locomotor response to amphetamine (AMPH) administration, a dopaminergic stimulant. This test evaluated sensitivity to the psychostimulants, in an attempt to unravel any potential alterations in dopamine signaling, as amphetamine increases synaptic dopamine release that acts in D1 and D2 receptors that alter PKA activation. With the PKA phosphorylation of PLPPR3 already established, we sought to investigate any possible behavioral alteration progressing from this axis.

The analysis of distance traveled in the OFT following amphetamine (AMPH) administration demonstrated a strong and significant effect of the drug on locomotor activity, while genotype and the interaction between drug and genotype had no significant effects. It should be noted here that WT mice as a group exhibited significant variability concerning AMPH-induced hyperlocomotion (8000-75000 cm) compared to PLPPR3 KO mice (17000-50000 cm). Analysis of the kurtosis and skewness value statistics of the four groups revealed that the KO AMPH group deviated from the other 3 groups. Specifically, the KO AMPH group showed a kurtosis value of 0.18, compared to values of 0.6-1 for the other groups, and a skewness value of 0.09, compared to values of 1.1-1.4 for the other groups. Despite these differences, AMPH elicits a pronounced increase in locomotion in both genotypes, and this effect appears to be independent of genetic differences associated with PLPPR3 KO (Figure 3.15).

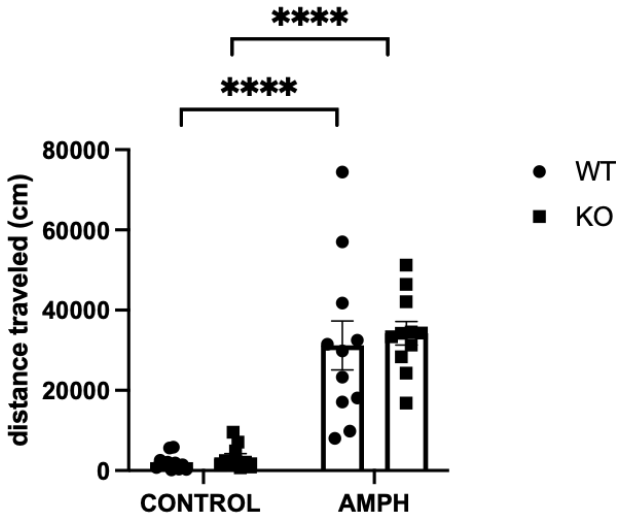


Figure 3.15 Amphetamine effects examined in the OFT. AMPH significantly increased locomotor activity compared to saline controls in both WT and KO mice ($p<0.0001$). No significant difference was observed between WT and KO mice after AMPH administration. WT control: n=11, WT AMPH: n=11, KO control: n=10, KO AMPH: n=11.

3.2. Molecular Results

3.2.1. Background

PLPPR3 and PTEN-Related Signaling

Previous studies have shown that PLPPR3 negatively regulates PTEN activity (Brosig et al., 2019), suggesting that its loss may influence key intracellular signaling cascades downstream of PTEN, such as the PI3K/Akt, GSK3, mTOR, and ERK pathways. Given this functional relationship, one of the major aims of our study was to investigate whether the absence of PLPPR3 in KO mice leads to alterations in these signaling pathways in vivo.

PKA-Dependent Regulation of PLPPR3 and Amphetamine Effects

As previously mentioned, PLPPR3 is phosphorylated at serine 351 by PKA, an event that enables its interaction with the scaffolding protein BASP1 (Kroon et al., 2024). This interaction is thought to influence presynaptic mechanisms, including vesicle release dynamics (Kroon et al., 2025, accepted manuscript) though the precise mechanisms remain unknown. Amphetamine increases dopaminergic transmission and activates PKA, providing a relevant tool to probe PKA-dependent phosphorylation of PLPPR3 in vivo. A second key objective of our study was to examine whether amphetamine treatment induces PKA-mediated phosphorylation of PLPPR3, and whether this affects the signaling capacity or interaction

networks at presynaptic sites. Exploring these questions could offer new insights into the presynaptic functions of PLPPR3 and its potential modulation by psychostimulant exposure.

3.2.2. Molecular Analysis of Key Signaling Pathways in the Prefrontal Cortex of Plppr3 Knockout Mice

Overview of Experimental Design

Our molecular analysis can be divided into two parts, the possible signaling differences of PLPPR3 KO compared to WT mice and the differences induced by AMPH treatment, including the PKA-induced phosphorylation of PLPPR3 and possible alterations in the downstream signaling pathways.

To investigate the molecular impact of PLPPR3 loss in the brain and the possible association with amphetamine-induced PKA activation, western blot analysis was performed on protein lysates from the prefrontal cortex (PFC) of WT and PLPPR3 KO three months old mice. Animals were either treated with saline serum or acutely with amphetamine (4 mg/kg) 1 hour before behavioral assessment, resulting in four experimental groups (WT-saline (SAL), WT-amphetamine (AMPH), KO-saline, KO-amphetamine; n=4 per group).

Validation of PLPPR3 Knockout

In parallel with the PCR genotyping results, it was also important to validate the PLPPR3 KO model in the protein level, via western blot. PLPPR3 protein was robustly detected in the PFC of adult WT mice and was absent in the KO samples, confirming successful deletion of the Plppr3 gene at the protein level (Figure 3.16).

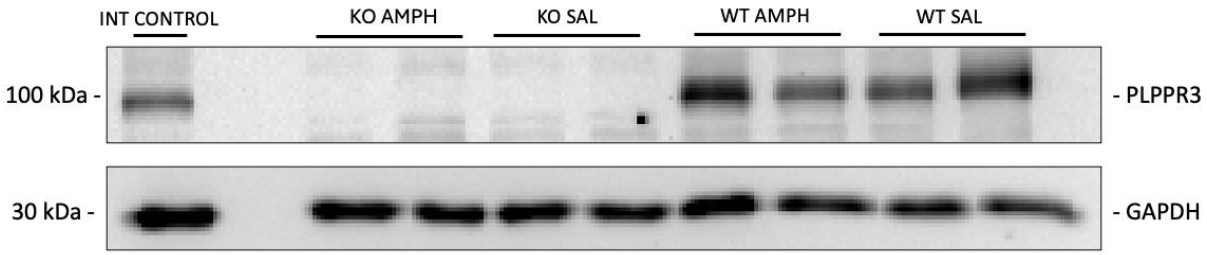


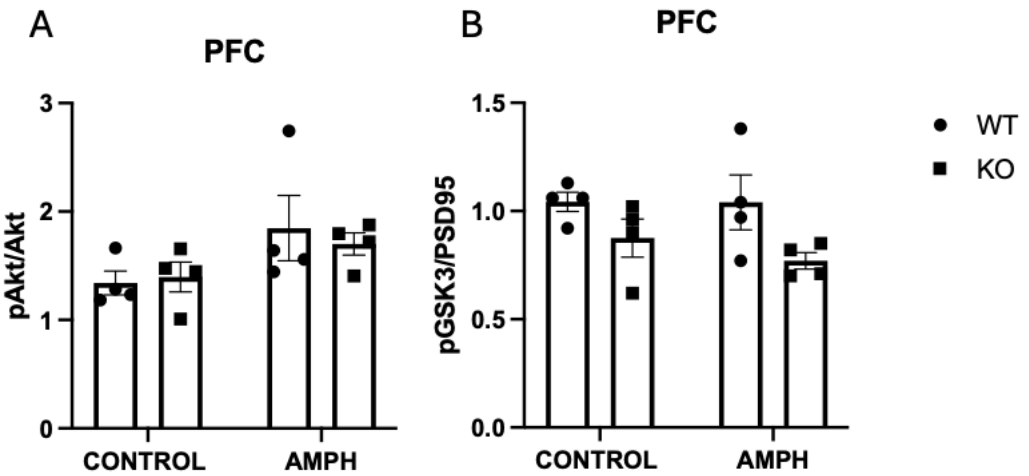
Figure 3.16 PLPPR3 KO validation in PFC samples of the amphetamine experiment. Western blot results for PLPPR3 expression in the hippocampus of WT and KO mice. No bands detected in the hippocampus of KO mice conferring the KO model and the correct genotyping assessment. INT CONTROL: a striatum protein sample from a WT mouse was used as internal control throughout the WB analysis.

Akt and GSK3 phosphorylation remains unchanged

The PI3K-Akt-GSK3 signaling axis is a central regulator of neuronal survival, plasticity, and cytoskeletal dynamics. Activation of Akt via phosphorylation at Ser473, typically triggered by upstream signals such as growth factors, neurotransmitters, and lipid mediators including LPA, enables the subsequent phosphorylation and inhibition of GSK3 at Ser21 (GSK3α) or Ser9 (GSK3β). This regulatory step plays a crucial role in modulating neuronal polarity, synaptic transmission, and protein synthesis (Hermida et al., 2017). Dysregulation of this pathway has been implicated in neurodevelopmental and neuropsychiatric disorders, including schizophrenia and mood disorders (Matsuda et al., 2019).

Given that PLPPR3 has been reported to suppress PTEN activity, a key inhibitor of the PI3K pathway (Brosig et al., 2019), we hypothesized that its deletion might alter Akt and GSK3 phosphorylation status. To test this, we analyzed levels of phospho-Akt (Ser473) and phospho-GSK3 in the PFC of both WT and PLPPR3 KO mice under basal (saline-treated) and amphetamine-stimulated conditions. Surprisingly, our results revealed no significant differences between genotypes or treatments, suggesting that PLPPR3 does not exert a major effect on this arm of intracellular signaling in vivo under the conditions tested.

No significant differences were observed in pAkt (Ser473) and pGSK3 (Ser9) levels between genotypes and were unaffected by amphetamine treatment (Figure 3.17).



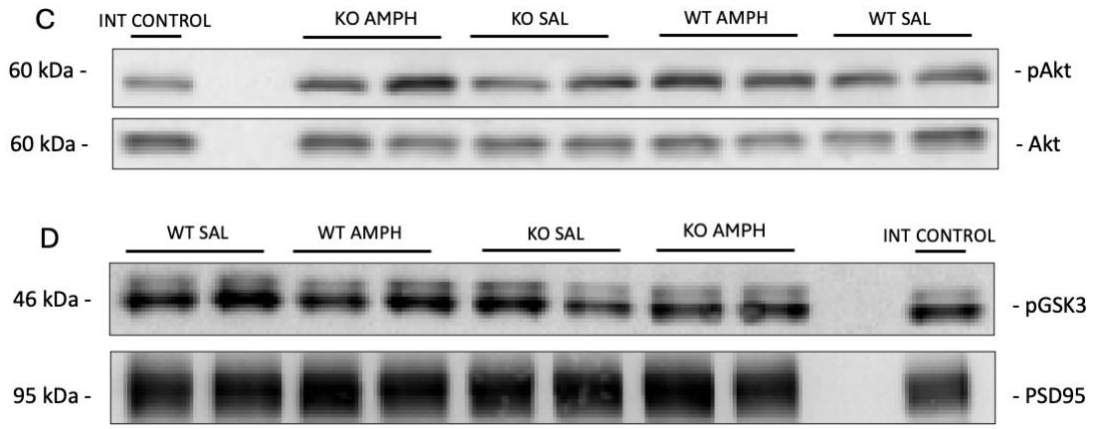


Figure 3.17 pAkt and pGSK3 expression in the prefrontal cortex of WT and KO mice after the amphetamine experiment. A. pAkt protein expression normalized by Akt protein expression. No statistical results were detected. B. pGSK3 protein expression normalized by PSD95 protein expression. No statistical results were detected. C. pAkt and Akt western blot bands. D. pGSK3 and PSD95 western blot bands. WT control: n=4, WT AMPH: n=4, KO control: n=4, KO AMPH: n=4.

ERK1/2 and CREB Phosphorylation Are Not Significantly Affected

The ERK-CREB signaling pathway is a critical regulator of neuronal plasticity, activity-dependent gene transcription, and stress adaptation. Activation of ERK1/2 (extracellular signal-regulated kinases) via the Ras-Raf-MEK cascade results in phosphorylation at Thr202/Tyr204. Once activated, ERK can phosphorylate the transcription factor CREB at Ser133, promoting the expression of genes involved in synaptic plasticity, memory consolidation, and neuronal adaptation (Albert-Gascó et al., 2020). This pathway is sensitive to dopaminergic modulation and is known to be activated by psychostimulants such as amphetamine, which engage both PKA- and MAPK-dependent mechanisms to induce ERK and CREB phosphorylation (Jia et al., 2021).

PLPPR3, may influence ERK-CREB dynamics via its known regulation by PKA (Kroon et al., 2024), and potential connections to LPA-mediated signaling cascades. To investigate this, we assessed ERK and CREB phosphorylation in the PFC of WT and PLPPR3 KO mice following saline or amphetamine treatment to determine whether loss of PLPPR3 perturbs this key signaling axis in vivo.

Levels of phosphorylated ERK1/2 (Thr202/Tyr204) and phosphorylated CREB (Ser133) showed no statistically significant differences between genotypes or treated groups (Figure 3.18). These findings suggest that the Ras-ERK-CREB pathway is not prominently affected by PLPPR3 deletion in the PFC under the conditions tested.

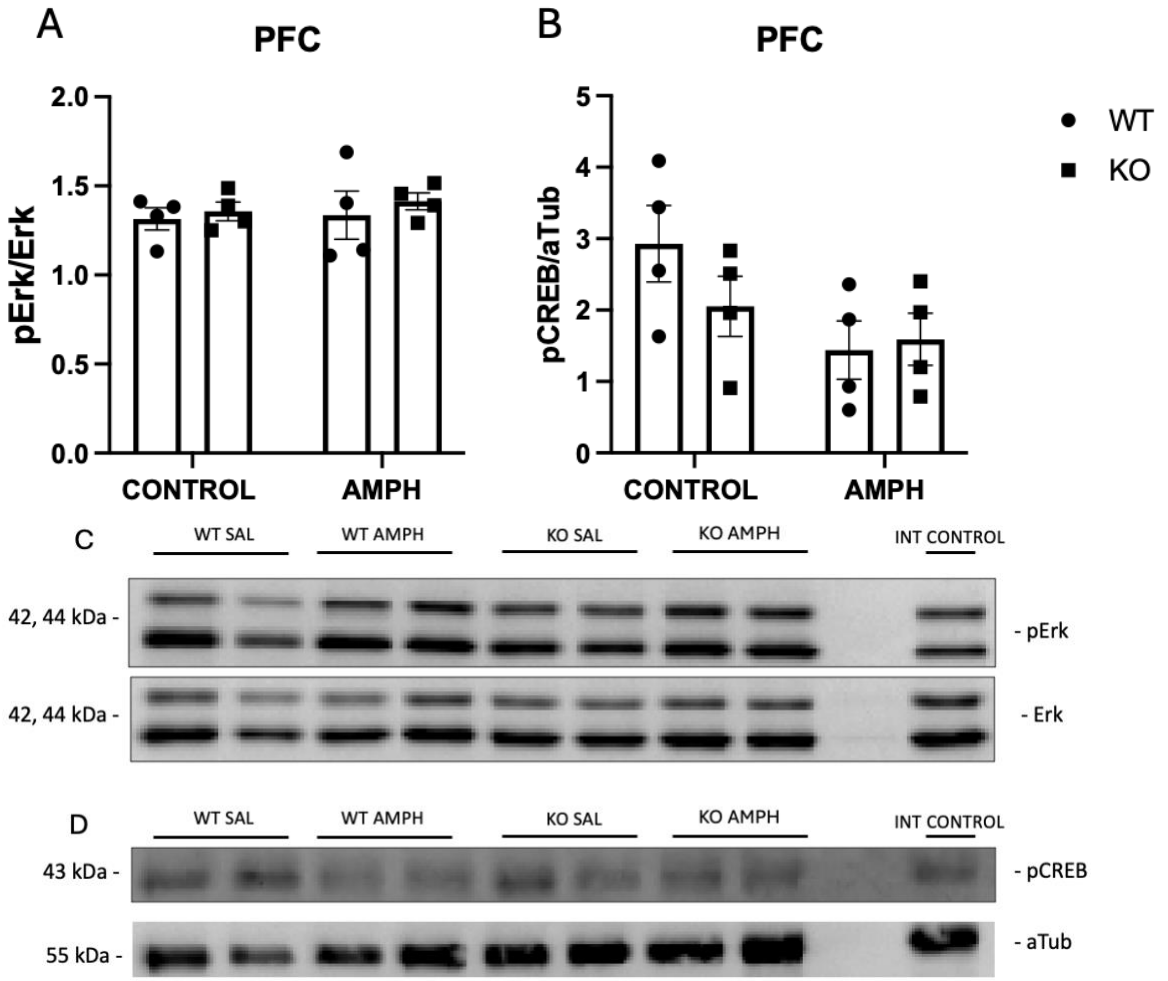


Figure 3.18 pErk and pCREB expression in the prefrontal cortex of WT and KO mice after the amphetamine experiment. A. pErk protein expression normalized by Erk protein expression. No statistical results were detected. B. pCREB protein expression normalized by a-Tub protein expression. No statistical results were detected. C. pErk and Erk western blot bands. D. pCREB and aTub western blot bands. No statistical results were detected. n=4 per group.

PLPPR3 KO effect on translational machinery

Ribosomal protein S6, when phosphorylated at Ser235/236, reflects the activation state of the mTORC1 pathway. mTORC1 integrates diverse upstream cues, including growth factors, metabolic status and intracellular signaling via the PI3K-Akt and ERK cascades, to control translational initiation and ribosome production (Goul et al., 2023). Phosphorylation of S6 (pS6) enhances the translation of specific mRNAs that are crucial for synaptic plasticity and remodeling, and its expression levels are dynamically modulated by neurotransmitters and psychostimulants such as amphetamine (Biever et al., 2015).

Given that PLPPR3 has been shown to suppress PTEN activity, a key brake on PI3K-Akt-mTOR signaling (Brosig et al., 2019), its deletion may alter mTORC1 activity and thereby affect downstream translational processes. To explore this possibility, we assessed levels of phosphorylated S6 in the PFC of WT and PLPPR3 KO mice under both control and amphetamine-treated conditions, probing for potential shifts in mTORC1-mediated signaling in vivo.

Analysis of pS6 revealed a significant increase in the control KO group compared to WT controls (Figure 3.19A and B). Total S6 levels were not significantly different across groups (Figure 3.19B). No significant changes in pS6 were observed following amphetamine treatment.

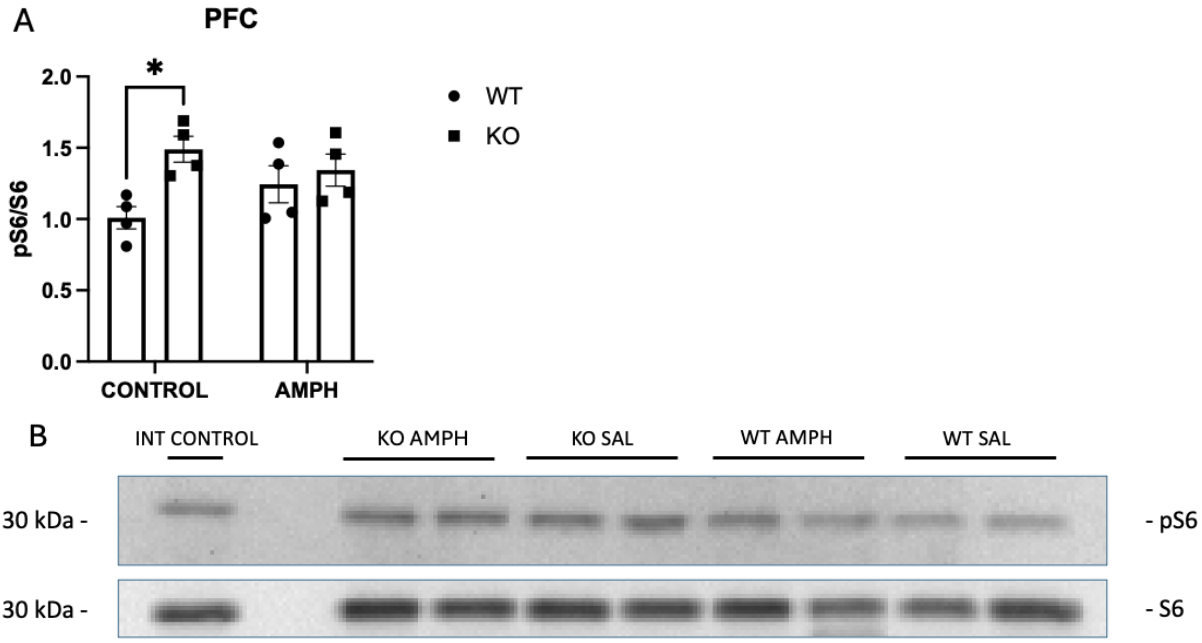


Figure 3.19 pS6 expression in the prefrontal cortex of WT and KO mice after the amphetamine experiment. A. pS6 was significantly increased in KO controls versus WT controls (p=0.0306), while amphetamine had no additional effect. B. Western blot protein band detection for S6 and pS6. n=4 per group.

Synaptic markers synaptophysin and PSD-95 are unaltered

To evaluate whether PLPPR3 deletion induces structural or functional changes at the synapse, we assessed the expression of two well-characterized synaptic proteins, postsynaptic density protein 95 (PSD95) and synaptophysin. PSD-95 is a major scaffolding molecule concentrated at excitatory postsynaptic sites, where it anchors glutamate receptors and organizes downstream signaling complexes essential for synaptic strength and plasticity. Alterations in PSD-95 levels have been linked to changes in synaptic density, receptor trafficking, and neurodevelopmental disorders (Coley & Gao, 2018).

On the presynaptic side, synaptophysin is a synaptic vesicle glycoprotein critical for maintaining vesicle pool stability and is widely used as a marker of presynaptic integrity (Gudi et al., 2017). By quantifying both PSD-95 and synaptophysin, we aimed to determine whether PLPPR3 loss disrupts synaptic organization in a region-specific or treatment-sensitive manner, potentially revealing downstream consequences of its presynaptic localization and signaling functions.

PSD-95 and synaptophysin expression, used as markers of synaptic density, showed no significant genotype- or treatment-related changes (Figure 3.20).

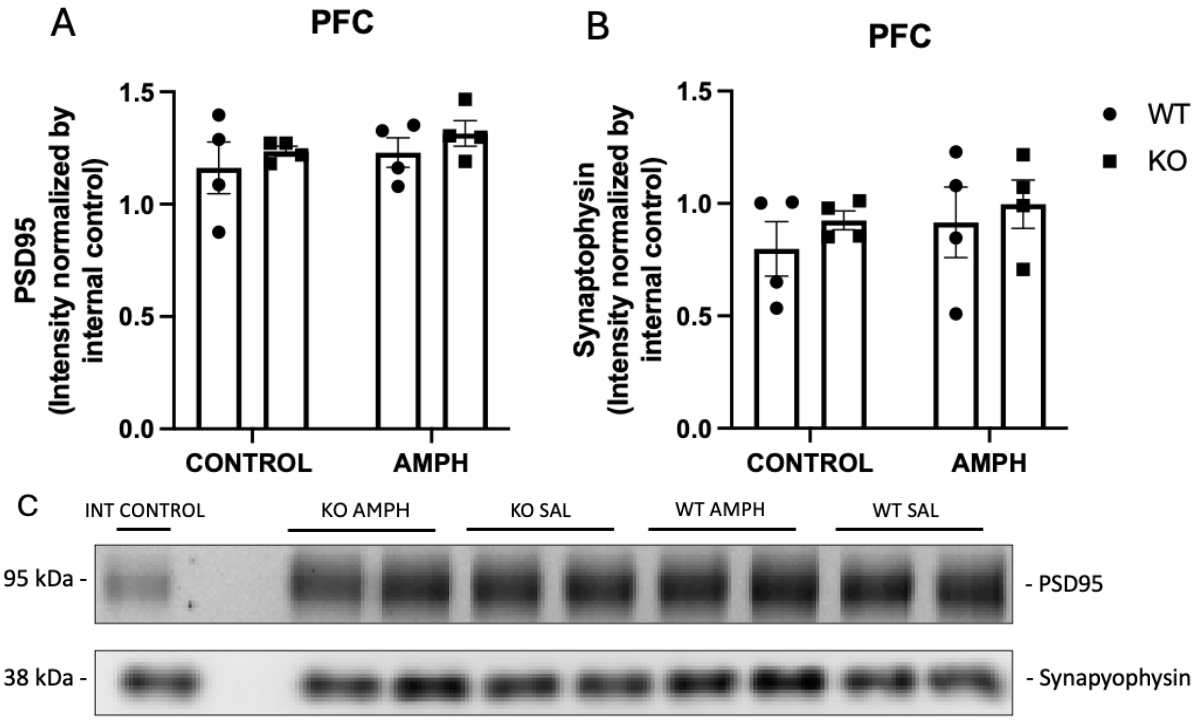


Figure 3.20 PSD95 and synaptophysin expression in the prefrontal cortex of WT and KO mice after the amphetamine experiment. A. PSD95 protein expression normalized internal control. No statistical results were detected. B. Synaptophysin protein expression normalized by internal control. No statistical results were detected. C. PSD95 and Synaptophysin western blot bands. n=4 per group.

Amphetamine has no significant effects in prefrontal cortex PLPPR3 expression

After analysis of the samples, amphetamine treated WT mice had similar PLPPR3 expression levels with untreated WT mice (Figure 3.21).

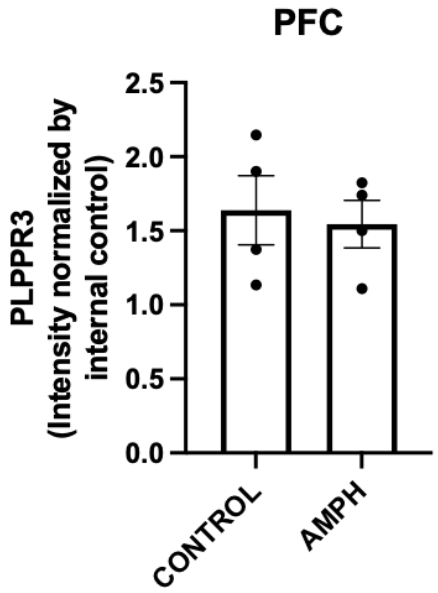


Figure 3.21 PLPPR3 protein expression in the prefrontal cortex of WT mice after saline and amphetamine treatment. No statistical differences were observed. n=4 per group.

Summary

The molecular analysis of the PFC revealed that deletion of PLPPR3 did not significantly affect phosphorylation levels of Akt, GSK3, ERK1/2, or CREB under either control or amphetamine-treated conditions. These findings suggest that major signaling axes downstream of LPA receptors, such as the PI3K-Akt-GSK3 and Ras-ERK-CREB pathways, remain largely unaltered in the absence of PLPPR3 at least in the PFC. However, a significant increase in phosphorylated S6 was observed in KO control animals compared to WT controls (Figure 5), a controversial result concerning PLPPR3 and PTEN interaction. Total S6 and the synaptic marker PSD-95 were unchanged, and expression of the housekeeping proteins GAPDH and α-tubulin remained stable across all groups, validating the normalization of protein levels. These findings point toward a selective modulation of translational signaling rather than broad disruptions across canonical signaling pathways in the PFC of PLPPR3 KO mice.

3.2.3. Molecular Analysis of Synaptosomal Protein Expression in the Hippocampus

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Overview

To assess potential synaptic alterations at the subcellular level, hippocampal synaptosome fractions were isolated from WT and PLPPR3 KO mice. Due to the specific tissue mass required

for successful synaptosomal preparation (30–40 mg of hippocampal tissue), only samples that met this criterion were included in the analysis. This resulted in the following group sizes: WT SAL (n=3), WT AMPH (n=3), KO SAL (n=3), and KO AMPH (n=3). Western blot analysis was performed to evaluate the expression of key synaptic and signaling proteins.

Assessment of synaptosomal fraction quality

For the assessment of our synaptosomal isolation from hippocampal tissue of WT and PLPPR3 KO mice, we used the presynaptic marker synaptophysin and the postsynaptic marker PSD-95 in a western blot analysis (Figure 3.22). PSD-95 expression was highly enriched in the LP1 fraction compared to other fractions, confirming the successful enrichment of postsynaptic density components in the synaptosomal preparation (Figure 3.22A). Synaptophysin was also predominantly detected in the LP1 fraction, consistent with the presence of intact presynaptic terminals within the isolated synaptosomes (Figure 3.22B). The pattern of distribution across fractions further validates the integrity of the synaptic isolation process. Both markers demonstrated reduced levels in cytosolic (S2) or nuclear (P1) fractions (not shown), confirming fractionation specificity. Comparable levels of PSD-95 and synaptophysin were observed between WT and PLPPR3 KO mice, indicating that synaptosomal integrity was not affected by the absence of PLPPR3.

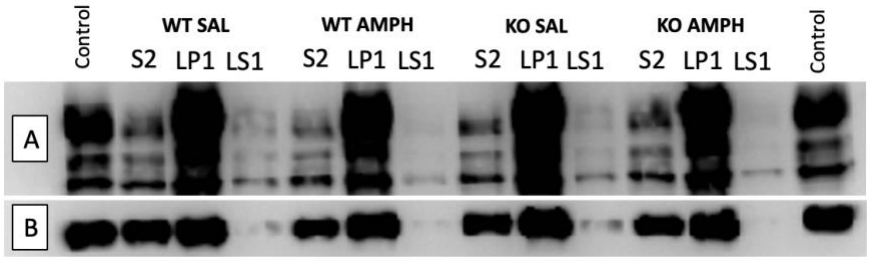

Figure 3.22 Western blot validation of synaptosomal fractions. A. Enrichment of postsynaptic marker PSD-95 and B. presynaptic marker Synaptophysin across subcellular fractions.

Phosphorylation Status of hippocampal PLPPR3 is Unaltered by Amphetamine

PLPPR3 is enriched at neuronal membranes and has been shown to localize at presynaptic sites. Its phosphorylation at S351 by PKA is thought to influence interactions with cytoskeletal regulators like BASP1 (Kroon et al., 2024). Since amphetamine elevates dopamine and subsequently PKA activity, we investigated both total PLPPR3 and its phosphorylated form in hippocampal synaptosomes to determine whether PLPPR3 signaling is modulated by acute dopaminergic stimulation at the synaptic level.
Phosphorylated PLPPR3 levels, normalized to total PLPPR3, showed no significant difference between control and amphetamine-treated groups (Figure 3.23). This suggests that acute

amphetamine exposure does not alter the phosphorylation status of PLPPR3 at the synaptic level.

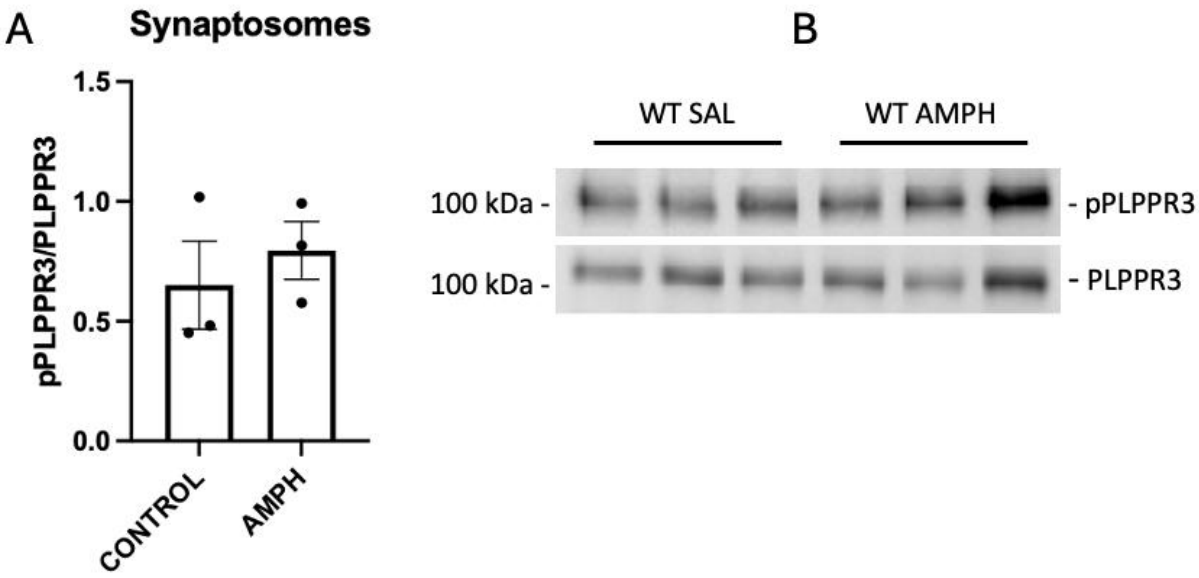

Figure 3.23 pPLPPR3 expression in hippocampal synaptosomes after the amphetamine experiment. A. Western blot quantification of phosphorylated PLPPR3 levels normalized to total PLPPR3. No significant differences were observed in response to amphetamine treatment. B. pPLPPR3 and PLPPR3 Western blot bands. WT control: n=3, WT AMPH: n=3

Synaptosomal Akt Signaling is Unaffected by Genotype or Treatment

While Akt signaling was already evaluated in the PFC to assess broader regional effects, its role at the synaptic level remains particularly relevant due to its local involvement in presynaptic plasticity, vesicle release, and activity-dependent signaling (Manning & Toker, 2017). By examining pAkt in hippocampal synaptosomes, we aimed to determine whether PLPPR3 deletion alters Akt activation specifically within the synaptic compartment, where spatially restricted signaling could contribute to functional changes not evident at the tissue level.

Akt phosphorylation remained unchanged across genotypes and treatments, with comparable pAkt/Akt ratios observed in all groups. These results imply that neither PLPPR3 loss nor amphetamine significantly impacts Akt activation in hippocampal synaptosomes (Figure 3.24).

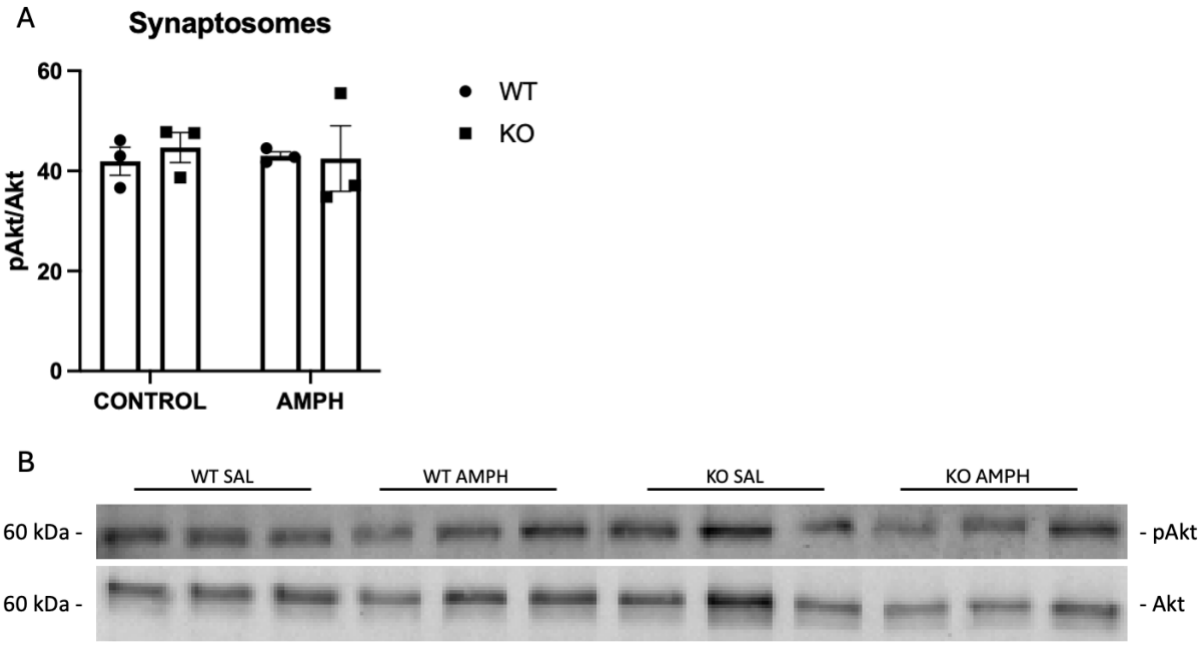

Figure 3.24 pAkt expression in hippocampal synaptosomes of WT and KO mice after the amphetamine experiment. A. Quantification of phosphorylated Akt (Ser473) normalized to total Akt levels. No significant differences were detected across genotypes or treatment groups. B. n=3 per group.

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pGluA1 Levels Show No Clear Genotype or Treatment Effects

Phosphorylation of the AMPA receptor subunit GluA1 at Ser831 and Ser845 plays a key role in regulating synaptic strength, receptor trafficking, and long-term potentiation (Banke et al., 2000; Mohanan et al., 2022). These phosphorylation events are modulated by kinases such as PKA and CaMKII, both of which can be activated downstream of dopaminergic or glutamatergic signaling (Nishi et al., 2000). Since PLPPR3 may influence intracellular pathways that converge on synaptic plasticity mechanisms, pGluA1 was examined to assess whether its phosphorylation status at the synaptic level is affected by PLPPR3 loss or amphetamine-induced signaling.

Phosphorylated GluA1 levels were quantified both relative to GAPDH and as absolute signal intensity. Across both metrics, there was no clear genotype- or treatment-dependent effect (Figure 3.25).

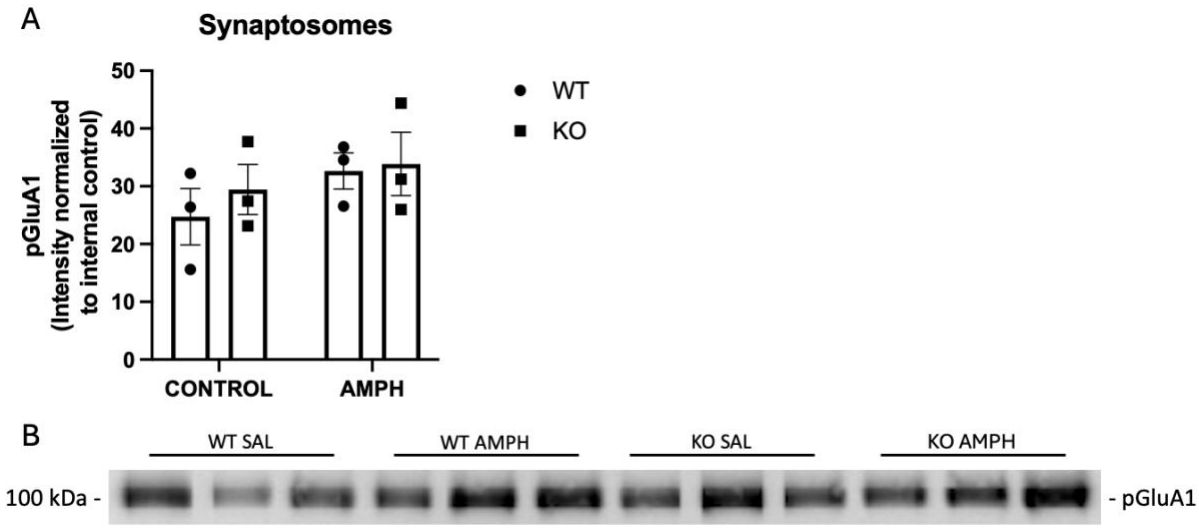

Figure 3.25 pGluA1 expression in hippocampal synaptosomes of WT and KO mice after the amphetamine experiment. A. pGluA1 levels normalized to internal control. B. pGluA1 western blot band signal. No statistically significant changes were found. n=3 per group.

BASP1 Expression Remains Stable Relative to PSD-95

BASP1 is a presynaptic protein known to interact with PLPPR3 in a phosphorylation-dependent manner, particularly at S351 via PKA activation (Kroon et al., 2024). Given its functional role in actin dynamics and vesicle trafficking at synaptic terminals, BASP1 serves as a valuable readout for PLPPR3-related presynaptic signaling. By examining BASP1 levels in hippocampal synaptosomes, we aimed to determine whether PLPPR3 deletion or amphetamine-induced phosphorylation modulates its expression or stability within the presynaptic compartment.

No significant differences in BASP1 levels normalized to PSD-95 were found across any condition, indicating that presynaptic plasticity markers remain stable despite PLPPR3 deletion or amphetamine administration (Figure 3.26).

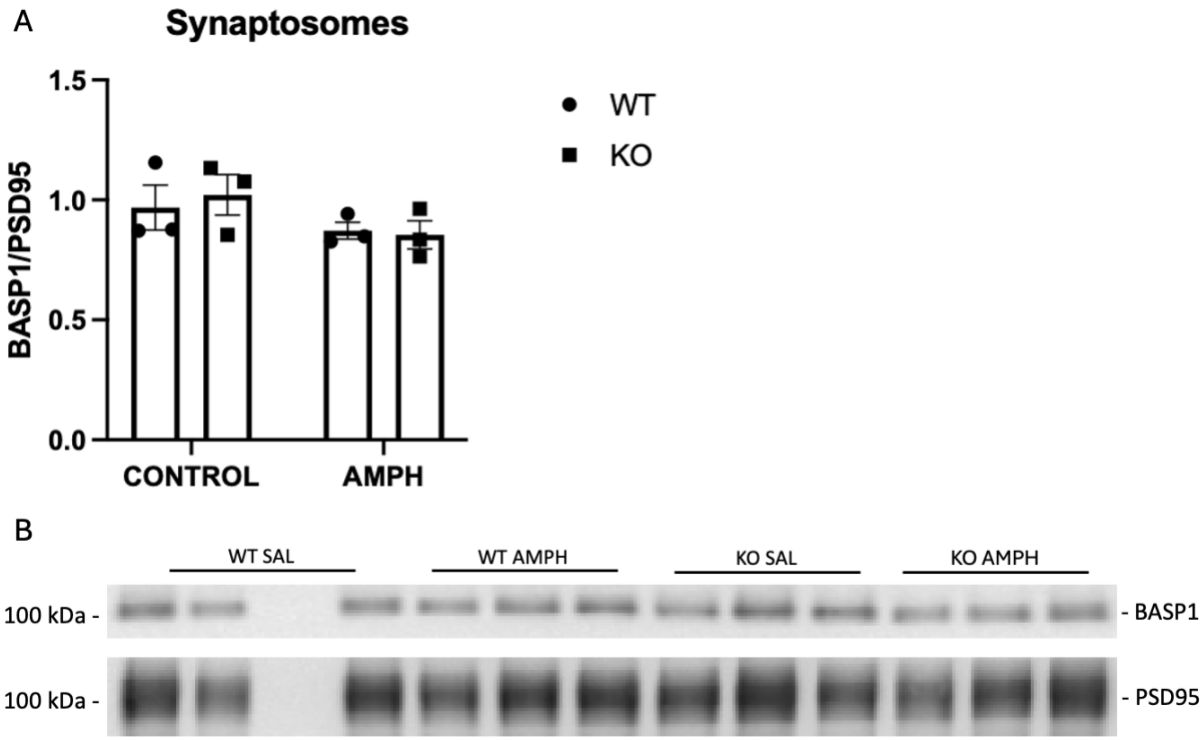

Figure 3.26 BASP1/PSD95 expression ratio in hippocampal synaptosomes of WT and KO mice after the amphetamine experiment. A. BASP1 levels normalized to PSD-95 were not significantly affected by genotype or treatment. B. BASP1 and PSD95 western blot bands. n=3 per group.

Summary

Collectively, these findings suggest that PLPPR3 deletion does not produce robust changes in synaptic signaling or structural markers in hippocampal synaptosomes, under basal or amphetamine-treated conditions.

Across both brain regions and subcellular compartments examined, our molecular analyses indicate that PLPPR3 deletion does not broadly disrupt canonical intracellular signaling pathways or synaptic protein composition. In the PFC, no significant differences were detected in the phosphorylation of key signaling proteins including Akt, GSK3, ERK1/2, and CREB, nor in the expression of synaptic markers such as PSD-95 and synaptophysin. An exception was a selective increase in pS6. In parallel, the hippocampal synaptosome analysis revealed no major genotype- or treatment-dependent changes in phosphorylation or expression of synaptic regulators such as pPLPPR3, pAkt, pGluA1, BASP1. These findings underscore a region- and compartment-specific profile of PLPPR3 function, potentially influenced by its differential expression levels across brain areas in 3-month-old mice. Taken together, the data suggest that PLPPR3 likely exerts its effects on signaling in a highly developmental stage-dependent and localized manner.

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4. Discussion

4.1. Summary of Main Findings

This study provides novel information on the behavioral and molecular changes of a PLPPR3 KO mouse model. Through a battery of behavioral assessment (OFT, EPM, MBT), we found that male PLPPR3 KO mice exhibited increased exploratory behavior, and a reduced anxiety-like behavior compared to male WT mice. In an attempt to establish a phenotypic connection between LPA and PLPPR3, we tested the ATX inhibitor PF8380 and its effects on behavioral tests of WT and PLPPR3 KO mice. Our results concerning the EPM test were not statistically significant, suggesting that more experiments or different behavioral tests are needed to reach conclusive results. In parallel, given the established phosphorylation of PLPPR3 by PKA and its purported role in regulating synaptic activity we sought to test whether a known CNS stimulant, amphetamine, that activates dopaminergic receptor-mediated PKA signaling can affect the PLPPR3 KO behavioral and molecular phenotypes. Amphetamine administration increased locomotion of both WT and PLPPR3 KO mice to a similar extent in the OFT. Molecular results using western blotting in PFC samples and in hippocampus synaptosomal samples showed no statistically significant results.

4.2. Behavioral Phenotype of PLPPR3 KO Mice

The behavioral results strongly support an anxiolytic-like phenotype in PLPPR3 KO mice. Compared to WT mice, KO animals exhibited increased locomotion and central zone exploration in the OFT (Figure 3.2), increased open arm time and entries in the EPM (Figure 3.5 and 3.6) and buried significantly less marbles in the MBT (Figure 3.7), indicating that PLPPR3 may play a regulatory role in emotional reactivity and compulsivity. Parallel work in the lab with PLPPR3 KO female mice has shown similar increased exploration and reduced anxiety-like behavior in PLPPR3 KO female mice compared to WT female mice (A. Dimoudi, diploma thesis, 2025). This suggests that the PLPPR3 KO behavioral phenotypes presented in this work are not gender specific.

Previous studies have largely focused on cellular and developmental roles of PLPPR3, mainly in axonal branching and thalamocortical guidance (Brosig et al., 2019; Cheng et al., 2016), while the behavioral study of a different PLPPR3 KO model by Cheng et al. detected a sensorimotor deficit in PLPPR3 KO mice. Notably, in this study, the authors did not report any data on novelty reaction or habituation in the OFT, nor data on EPM behaviors. However, they showed that PLPPR3 KO male mice do not exhibit any learning deficits using a Morris Water Maze (MWM) test and, no deficits in motor activity as judged by velocity measurements in the MWM test (Cheng et al., 2016). Furthermore, this study has excluded any difference in

normal motor development and strength at early postnatal ages (P15-P30) of PLPPR3 KO mice (Cheng et al., 2016). Our work consistently showed increased locomotion of PLPPR3 KO mice in the OFT (Figure 3.4). Importantly the increased locomotion was observed during both the reaction to novelty phase (i.e. 0-30 min of the test) and the habituation phase (i.e. 30-60 min of the test), suggesting also some form of non-associative learning deficit in the PLPPR3 KO mice.

Concerning the sensory discrimination deficits described in the PLPPR3 KO mouse model of Cheng et al., we have tested the ability of the current PLPPR3 KO model to distinguish texture coarseness in chambers covered with sandpaper of different grades (Labrakakis and Leondaritis, unpublished), but with inconclusive results so far. Interestingly, the experimental design for the PLPPR3 KO models of Cheng et al., 2016 and Brosig et al., 2019, have targeted the same region of the PLPPR3 gene (exon 1), but they have followed different approaches. Brosig et al. used a CRISPR/Cas9 mediated non-homologous end-joining (NHEJ) to target PLPPR3 exon 1 (Brosig et al., 2019), while Cheng et al. produced a PLPPR3 KO mice line after mating mice with flanked PLPPR3 exon 1 with LoxP sites, with a Del-Cre mice line (Cheng et al., 2016). CRISPR/Cas9 is practically a pair of molecular scissors, whereas Cre-LoxP is a molecular switch. This difference may be a factor to take into consideration when comparing and discussing the two PLPPR3 KO models in terms of baseline behavioral phenotypes. For example, is there a possibility that the anxiolytic-like phenotype we detected for the PLPPR3 KO model of Brosig et al in adult 2–3-month-old mice is related to the developmental phenotype of mistargeted thalamocortical axonal projections in the barrel cortex (or other cortical areas) in the Cheng et al., 2016 PLPPR3 KO model? The lack of detailed assessment of behavioral phenotypes of the Cheng et al PLPPR3 KO model does not provide any insight on this question. We searched the literature whether deficiencies in early developmental thalamocortical projections are connected to anxiolytic phenotypes in the adult, and we have found no evidence for any such connection so far. Regardless, to formally answer this question, a proposed experiment could be to induce a PLPPR3 knockout or knockdown in mice after postnatal development where thalamocortical targeting has been established. That will give us the answer on how fundamental is PLPPR3 for these behavioral phenotypes and whether its function is solely during development. This is an important question since recent studies from our lab have suggested that PLPPR3 may have roles in synaptic transmission in established synapses (Polyzou et al., 2024; Kroon et al., 2024; Kroon et al., 2025, accepted manuscript; Polyzou et al., 2026). In parallel, our current ATX inhibition tests in adult PLPPR3 KO mice could also be relevant to address this question. This hypothesis is discussed in the following sections.

Comparison of the PLPPR3 KO phenotype with the phenotypes of other PLPPRs is also very revealing. PLPPR4 is the most studied PLPPR protein. PLPPR3 and 4 share a similar structure with a long intracellular C-terminal domain that participates in interaction with signaling proteins like PTEN (Brosig et al., 2019) ERM proteins (Cheng et al., 2016), PP2A (Liu et al., 2016)and calmodulin (Tokumitsu et al., 2010), but their localization and expression patterns

are very different (Fuchs et al., 2022). We wanted to compare our behavioral results with those of PLPPR4 KO models (Endle et al., 2022; Schneider et al., 2018; Thalman et al., 2018; Tüscher et al., 2024) in order to figure out any similar or conflicting patterns, with a focus on anxiety-like phenotypes. PLPPR4 KO mice displayed increased locomotion and distance traveled (Schneider et al., 2018) and PLPPR4 +/R346T mice, which model humans with an SNP inactivating PLPPR4-mediated LPA uptake, also showed increased hypermobility (speed) (Thalman et al., 2018), with PLPPR3 mice also displaying increased distance traveled. However, when testing the ratio of center/periphery in OFT they saw a decrease for PLPPR4 KO and PLPPR4 +/R346T mice (Schneider et al., 2018; Thalman et al., 2018; Tüscher et al., 2024), while our results with PLPPR3 KO mice showing a tendency for increased presence in the center zone. Adding the decrease in vertical activity (rearings) and the increased grooming, Schneider et al. characterized an increased anxiety-like model that they linked to ASD, while Tüscher et al. characterized their PLPPR4 +/R346T mice with anxiety/depressive syndrome. Meanwhile, our results together with the increased presence in the open arms of the EPM and the fewer marbles buried in the MBT are most consistent with a decreased anxiety-like model. Thus, it would appear that PLPPR4 and PLPPR3 exert opposing effects in anxiety-related behaviors.

A notable observation in our study is the effect of acute stress in normalizing the behavioral phenotypes of PLPPR3 KO. After exposure to acute restraint stress, KO mice no longer exhibited the reduced anxiety-like behavior on both EPM (Figure 3.11 and 3.12) and OFT (Figure 3.9). While stress exposure disrupted the low-anxiety phenotype in KO mice, it did not induce exaggerated responses, while WT mice had almost similar responses even though they were not statistically relevant. This suggests that the phenotype can be a difference to the innate anxiety levels and not the phenotype of resilience to exogenous stressors.

In an attempt to further characterize our PLPPR3 KO mice behavioral model, it is noteworthy to discuss the dopamine transporter 1 (DAT) KO model that has been associated with an attention deficit hyperactivity (ADHD)-related phenotype (Kim et al., 2024). DAT is an integral membrane protein with 12-transmembrane regions that removes dopamine from the synaptic cleft into presynaptic compartments and it is also the target of stimulants like cocaine and amphetamine (Giros et al., 1996). Interestingly, DAT is one of a handful of transmembrane proteins known to be able to induce filopodia as PLPPR3 (Caltagarone et al., 2015; Fuchs et al., 2022). DAT KO mice exhibit hyperactivity, with increased distance traveled, velocity and decreased percentage of immobility and no signs of habituation over time in an OFT compared to WT mice (Fox et al., 2013). DAT KO mice also bury less marbles compared to WT mice in a MBT (Fox et al., 2013). In another study on DAT KO mice, except from increased distance traveled, DAT KO mice had increased presence in the center zone of an OFT and no alteration of vertical activity (Spielewoy et al., 2000). Furthermore, in a dopamine transporter deficiency syndrome (DTDS) model with two DAT mutations, EPM analysis identified an increase in time spent in the open arms and decreased time spent in the closed arms (Herborg et al., 2023). The effects of amphetamine in DAT models are contradictory and

indicate an alleviation of the hyperactivity (Gainetdinov et al., 2001; Giros et al., 1996; Herborg et al., 2023). Cumulatively, these phenotypes, except amphetamine experiments, are present in our model suggesting the possible interaction of PLPPR3 with DAT regulation and a possible relevance for ADHD. To provide stronger connections of PLPPR3 deficiency to ADHD phenotypes we are planning to use additional behavioral tests, including attention (i.e. Attentional Set-Shifting Task) and memory (i.e. Novel Object Recognition) assessment.

4.3. Response of PLPPR3 KO mice to dopaminergic stimulation

Amphetamine is known to increase extracellular dopamine levels, activate PKA signaling and promote locomotion in rodents, among other effects. Despite the established phosphorylation of PLPPR3 by PKA at S351 (Kroon et al., 2024), from our results there is not a strong amphetamine-induced PKA-dependent PLPPR3 S351 phosphorylation in vivo, at least in hippocampus synaptosomes, although in primary neurons, forskolin, a direct PKA activator, induces robust S351 phosphorylation of PLPPR3 (Kroon et al., 2024; Polyzou et al., unpublished data). It is likely though that this phosphorylation event may be more evident in other brain areas which are currently being tested in our lab.

PLPPR3 KO mice displayed a normal hyperlocomotion response to amphetamine, similar to WT. This finding suggests that PLPPR3 is not required for the expression of dopamine-dependent motor stimulation. This response to amphetamine may also indicate that while PLPPR3 possibly modulates aspects of basal emotional behavioral, e.g. anxiety like behavior, it does not significantly influence the acute dopaminergic activation of locomotor circuits, with the dosage of amphetamine 4 mg/kg being under discussion. Similar results have been obtained in additional experiments in the lab (A Dimoudi, 2025). A consistent finding in all amphetamine treatment experiments was a stark differential distribution of amphetamine-induced hyperlocomotion responses in WT vs KO mice (Figure 3.15). Skewness and Kurtosis test values for the KO AMPH are close to 0 meaning that the normal distribution has low levels of possible outliers, compared to WT AMPH mice that have an increased score for non-canonical distribution and higher possibility of outliers. This distribution of KO mice after AMPH is noteworthy. Taking these results into account, amphetamine dose-dependent tests would be an important step to understand if lower dosages could be more efficient to see the interaction between the PLPPR3 KO model and amphetamine.

Molecular analyses in PFC lysates did not reveal robust differences in the major intracellular signaling pathways of PI3K-Akt and ERK-CREB suggesting that PLPPR3 deletion does not affect these cascades under basal or amphetamine-induced conditions. However, the mTOR pathway was activated in the PFC of KO mice compared to WT mice, as phosphorylation of S6 was found to be increased in KO PFC, with no further changes induced by amphetamine (Figure 5). Previous studies in cell lines and primary neurons have suggested that PLPPR3

inhibits PTEN which acts as a brake in the Akt/mTOR pathway (Brosig et al., 2019). As such, deletion of PLPPR3 should derepress PTEN and result in a decrease of Akt/mTOR activity and thus decrease of pS6. Our finding of increased pS6 may suggest a complex interaction of PLPPR3 with the Akt/mTOR pathway in vivo. Additionally, it is likely that signaling responses may exhibit area-dependent specificity since expression of PLPPR3 varies substantially in adult CNS areas (Polyzou et al., 2024). In further studies in the lab, analyses of striatum, amygdala and hippocampus samples under basal and amphetamine-induced conditions has shown differences in the expression levels of PLPPR3, pS351-PLPPR3, pGluA1 and pDARPP52 in some tissues. Those results are promising to identify an area-specific signaling role of PLPPR3. Besides PTEN, PLPPR3 interacts also with RDX, an ERM protein (Cheng et al., 2016) and we tested basal levels and phosphorylation of ERM in western blot analysis of the PFC tissue samples. However, our results were inconclusive because of poor performance of the antibodies that were used for ERM and phospho ERM detection (results not shown).

In addition to PFC sample analysis, knowing that PLPPR3 is localized in the presynaptic compartment, we isolated synaptosomal fractions from hippocampal samples of the same amphetamine experiment. The main reason for using hippocampus samples was the quantity of tissue to suffice for proper fractionation and the confirmation of detected signals in the work of Polyzou (Polyzou et al., 2024). Subsequent studies may test other areas like striatum samples which may be more informative. No changes were observed in the synaptic fractions in basal or amphetamine-stimulated conditions. The combined molecular results depict that the effects of PLPPR3 deletion on the pathways may be area-restricted or transient, requiring investigating more brain areas and perhaps more precise temporal or subcellular resolution.

4.4. Response of PLPPR3 KO mice to acute ATX inhibition

As previously mentioned, LPA modulation is crucial for several of the PLPPR phenotypes. Specifically, ATX inhibition or LPAR2 knockout can normalize PLPPR4 KO phenotypes to WT levels (Endle et al., 2022; Trimbuch et al., 2009; Thalman et al., 2018; Tüscher et al., 2024). PF8380 was the common ATX inhibitor tested in all the above experiments, and it is also the one we tested in our attempt to underline a connection between our behavioral PLPPR3 KO model and LPA.

ATX, also known as ecto-nucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2), is a crucial secreted enzyme responsible for the vast majority of extracellular LPA production. ATX primarily functions as a lysophospholipase D that catalyzes the hydrolysis of LPC and other lysophospholipids into LPA. While other pathways can produce LPA, ATX is recognized as the predominant enzyme for LPA generation in various biological fluids, including blood plasma/serum, CSF) and other biological fluids (Herr et al., 2020). In essence, ATX acts as the master switch for controlling extracellular LPA levels, making it a highly attractive therapeutic

target for conditions where excessive LPA signaling is detrimental. PF-8380 is a highly effective and widely used type I ATX inhibitor that occupies the catalytic site and mimics binding of LPC. PF-8380 appears to penetrate the BBB and achieve effective concentrations in the brain and CSF (Joshi et al., 2021), making it a valuable tool for studying the role of brain ATX-LPA signaling in various CNS conditions, including those related to anxiety.

Administration of the ATX inhibitor PF8380 did not affect EPM anxiety-related behaviors in WT or KO mice, suggesting that acute blockage of LPA synthesis does not change responses in the EPM and is insufficient to modify the behavioral phenotype caused by PLPPR3 loss. This contrasts with studies in PLPPR4 KO mice, where ATX inhibition normalized behavioral abnormalities (Thalman et al., 2018). It is likely that larger groups or additional tests (e.g. MBT PLPPR3 phenotypes) may be required to see an effect by acute ATX inhibition.

LPA has been highlighted as a novel mediator of mood behavior by regulating synaptic neurotransmission and plasticity. On the one hand, acute administration of LPA was identified to induce anxiety- and depressive-like phenotypes both in rats (Castilla-Ortega et al., 2014) and mice (Yamada et al., 2015). On the other hand, chronic intracerebroventricular (ICV) infusion of LPA in WT mice results in increased exploration, reduced anxiety levels while improving spatial working memory (Rosell-Valle et al., 2021). Many studies have suggested a complex interplay between LPA, LPARs and anxiety-related behaviors (Li & Li, 2024). For example, LPAR1 null mice tested in OFT and EPM exhibited increased anxiety-like behavior and decreased exploration, with impaired spatial memory in MWM (Santin et al., 2009). Similar LPAR1 null phenotype of decreased exploration and increased anxiety-like behavior and decreased working memory were detected using a hole-board test (Castilla-Ortega et al., 2010). Specifically, the maLPA1 KO mouse model has been proposed to exhibit a mixed anxiety-depression phenotype (Moreno-Fernández et al., 2018) which can be partially recapitulated upon acute administration of LPAR1 antagonists (Moreno-Fernández et al., 2018), suggesting that LPA, primarily via LPAR1, acutely controls these behaviors. Opposing the LPAR1 KO, LPAR5 KO mice showed an anxiolytic phenotype (Callaerts-Vegh et al., 2012). Increased visits in the center and decreased thigmotaxis in OFT, combined with increased entries in the open arms of the EPM give an opposite phenotype compared to LPAR1 KO mice and are very similar to our PLPPR3 KO model.

As mentioned previously, the PLPPR4 phenotypes were normalized after double KO of PLPPR4 and LPAR2, placing LPA/LPAR2 regulation in the center of PLPPR4 phenotypes (Schneider et al., 2018). The known function of PLPPR4 is as a postsynaptic uptake mechanism, removing LPA from interacting with presynaptic LPAR2. Can PLPPR3, which is localized in the presynaptic compartment, have a similar function but interact with another LPAR and not LPAR2? Previous experiments have suggested that PLPPR3 can also increase uptake of LPA in cells but with different specificity compared to PLPPR4. Transfected HEK293T cells with PLPPR3-flag or PLPPR4-flag plasmids displayed a significant and comparable increased LPA uptake compared to control HEK293T cells. However, only those transfected with PLPPR3,

and not with PLPPR4, had increased MAG, the LPA dephosphorylation product, uptake, that could imply a different mechanism of lipid uptake for the two PLPPRs (Polyzou et al., 2024). An alternative mechanism of PLPPR3 at the presynapse, which may not be mutually exclusive with the LPA transport activity, relates to its effects on synaptic vesicle release. Deletion of PLPPR3 in primary neurons results in increased synaptic vesicle release, a phenotype which is normalized by wild-type PLPPR3 but not a mutant that is defective in PKA-dependent S351 phosphorylation (Kroon et al., 2025, accepted manuscript). Although this mechanism suggests that PLPPR3 may directly control presynaptic activity in terms of neurotransmitter release, it is not known yet if it relates to LPA transport activity while it has been studied only in primary neuron cultures and not in vivo. One interesting future experiment would be to investigate whether there are defects in neurotransmitter release or levels in different brain areas of PLPPR3 KO mice that may relate to the behavioral phenotypes established in this work.

4.5. Conclusions and future directions

In summary, our data demonstrate that PLPPR3 deletion leads to an anxiolytic-like behavioral phenotype, partially reversed by acute stress, and that this phenotype is not modified by acute administration of amphetamine or ATX inhibition. Molecularly, PLPPR3 appears to have a limited effect on canonical intracellular signaling pathways in the PFC or hippocampal synaptosomes, under the tested conditions. Future work should include larger molecular datasets and region-specific phospho-proteomic profiling to outline the neural substrates of PLPPR3-dependent behavior. Investigating PLPPR3 interactions with BASP1, dopamine transporters, and other presynaptic regulators may further clarify its role in modulating neurotransmission and emotional regulation. Ultimately, understanding the precise contributions of PLPPR3 to synaptic signaling and behavioral regulation may inform novel therapeutic approaches for neuropsychiatric conditions involving emotional dysregulation, including anxiety, ADHD, and schizophrenia.

5. Literature

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