Mapping of the Akt and Beta-arrestin Interface for Drug Development

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Pharmacology and Toxicology
University of Toronto

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2019

Abstract

G-protein-coupled receptors (GPCRs) are well-known therapeutic targets, and it has been demonstrated that they can signal both via guanine nucleotide-binding proteins (G proteins) signaling and arrestin signaling. Dopamine (DA) and dopamine receptors, which are GPCRs, are involved in numerous neuropsychiatric disorders. Notably, dopamine D2 receptor (D2R) signaling is targeted by drugs used for mental illnesses like bipolar disorder (BP) and schizophrenia (SZ).

Indeed, a signaling pathway involving arrestin signaling is targeted by both lithium and antipsychotic medications. This pathway involves a modulation of glycogen synthase kinase 3 (GSK-3) by D2Rs via a complex formed of Akt (also called protein kinase B or PKB), β-arrestin 2 (βArr2) and protein phosphatase 2A (PP2A). The aim of this project is to further elucidate this signaling pathway by studying the direct interaction between two of its components, the proteins Akt and βArr2, in view of possible drug development.
Acknowledgments

I would like to thank Dr Jean-Martin Beaulieu, my supervisor, for giving me the opportunity to work and study in his lab. These last two years have been full of challenges that allowed me to learn and grow so much as a scientist as well as a person and I am thankful for all of it. Thanks also go obviously to Dr Oliver Ernst, my advisor: collaborating with your lab was a highlight of my MSc experience and allowed me to expand my horizons even more. Thank you to Dr Leonardo Salmena for accepting to be assessor of this thesis, and to Dr Ali Salahpour for accepting to be on my committee; your support is greatly valued and much appreciated. Ghazal Fakhfouri, graduated PhD student in our lab, deserves many thanks for her help not only in conducting BRET experiments but in teaching and discussing experiments and various aspects of the project, including all the preliminary data coming from her own work. I have to thank also Kyumhyuk “Peter” Kim, MSc student and collaborator in Ernst’s lab, for his collaboration on this project, his help in protein expression and purification and his optimisation of these protocols, all of which were essential components of this whole project. Aleksandra Marakhovskaia, PhD student in our lab, deserves a special recognition for her constant guidance and support throughout the whole project. Thank you also to all Beaulieu lab members for their help and advice throughout my MSc. To all, thank you for everything. None of this would have been possible without all of you.

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# Table of Contents

Abstract .......................................................................................................................... ii

Acknowledgments ......................................................................................................... iii

Table of Contents ........................................................................................................... iv

List of Tables .................................................................................................................. viii

List of Figures ................................................................................................................ ix

List of Abbreviations ...................................................................................................... xi

Section 1 Introduction .................................................................................................... 1

1 GPCRs .......................................................................................................................... 1

1.1 A brief GPCR signaling overview .......................................................................... 1

1.2 Biased signaling of GPCRs .................................................................................... 3

2 D2R ............................................................................................................................. 4

2.1 D2R importance in neuropsychiatric disorders ..................................................... 5

2.1.1 Bipolar disorder and schizophrenia ................................................................. 5

2.1.2 D2R implication in BP and SZ ......................................................................... 6

2.2 D2R signaling ......................................................................................................... 6

2.2.1 D2R signaling .................................................................................................. 6

2.2.2 D2R signaling to GSK3 via an Akt-βarr2-PP2A complex ................................ 9

3 Arrestins and βarr2 ..................................................................................................... 13

3.1 General roles and functions of arrestins ................................................................. 13

3.2 Arrestin structure and activation .......................................................................... 15

4 Akt ............................................................................................................................... 16

4.1 General roles and functions of Akt ....................................................................... 16
4.2 Akt Structure and Activation ................................................................. 19

5 Preliminary results concerning the Akt-βarr2 Interaction ........................................ 21
  5.1 Active pAkt has a better affinity for βarr2 than inactive Akt ................................ 21
  5.2 Identification of two putative interfaces of interaction on βarr2 .......................... 21
  5.3 Confirmation of specificity for βarr2 ................................................................... 22
  5.4 TATPEP does not affect other aspects of the D2R-GSK3 signaling pathway ............ 22

6 Akt-βarr2 Interaction Project ............................................................................ 23
  6.1 Project rationale ................................................................................................ 23
  6.2 Research Hypotheses ....................................................................................... 23
    6.2.1 Phosphorylation sites of Akt can lead to the interaction site of Akt with βarr2 ... 23
    6.2.2 The PH domain of Akt may present an interaction site of Akt with βarr2 ....... 24
  6.3 Specific Objectives ......................................................................................... 24
    6.3.1 Investigation of the effect of different phosphorylation sites of Akt on its interaction with βarr2 ................................................................. 24
    6.3.2 Investigation of a possible interaction site on the Akt PH domain ................. 25
    6.3.3 Development of a split-luciferase NanoBiT assay for future screening of compounds ........................................................................................................ 25

Section 2 Materials and Methods ........................................................................ 27

7 Cloning ............................................................................................................. 27
  7.1 Cloning Methods ............................................................................................ 27
  7.2 List of constructs ............................................................................................. 27

8 Recombinant Protein Expression and Purification .............................................. 30
  8.1 GST-βarr2 and GST expression and purification .............................................. 30
  8.2 Mutated Akt constructs expression test ........................................................... 31
  8.3 Akt1-PH123 and βarr2 (Arr3 untagged) expression and purification ..................... 32
9 Pulldown Assays ...................................................................................................................... 32
  9.1 Pulldown assays with recombinant βarr2 ........................................................................ 32
  9.2 Pulldown assays with recombinant βarr2 and Akt1-PH123 ............................................ 33
10 Co-Immunoprecipitation (Co-IP) ....................................................................................... 33
11 Suspension BRET ................................................................................................................. 34
12 Split-luciferase NanoBiT Assay ........................................................................................... 35
  12.1 Design of the constructs ............................................................................................... 35
  12.2 Split-luciferase NanoBiT assay ..................................................................................... 36

Section 3 Results .......................................................................................................................... 37
13 In vitro studies of the Akt-βarr2 interaction ........................................................................ 37
  13.1 Active pAkt seems to have a better affinity for βarr2 than inactive Akt ......................... 37
  13.2 Optimisation of expression and purification of βarr2 .................................................... 38
  13.3 Expression of Akt constructs is favored by removal of the PH domain ......................... 39
    13.3.1 Cloning of pseudo-phosphorylated mutants of Akt .............................................. 39
    13.3.2 Expression test of Akt mutants constructs ............................................................ 39
  13.4 Expression and purification of Akt PH domain ............................................................... 41
  13.5 Recombinant Akt PH domain and βarr2 show a weak interaction in vitro ................... 41
14 Co-IP studies of the Akt PH domain and βarr2 interaction ................................................ 42
  14.1 Design and cloning of βarr2 and Akt constructs ............................................................ 42
  14.2 Akt1-PH48 and Myr-ΔPH-Akt1 interact with βarr2-sp1 ................................................ 46
  14.3 Confirmation of the PH domain interaction with βarr2 ................................................ 47
15 BRET studies of the Akt PH domain and βarr2 interaction ................................................ 48
  15.1 Akt1-PH48, but not Myr-ΔPH-Akt1, interacts with βarr2-sp1 ......................................... 48
  15.2 Confirmation of the PH domain interaction with βarr2 ................................................ 48
  15.3 The PH domain of another Akt isoform, Akt2, also seems to interact with βarr2 ......... 50
15.4 TATPEP can disrupt the interaction between Akt-PH domain and βarr2 ...............51
16 Split-luciferase NanoBiT assay ...........................................................................52
16.1 General considerations and design of constructs .............................................52
16.2 Protocol confirmation and orientation test .....................................................53

Section 4 Discussion .................................................................................................56
17 Discussion of results ............................................................................................56
17.1 In vitro studies ..................................................................................................56
17.2 Co-IPs ..............................................................................................................57
17.3 BRET assays ...................................................................................................58
17.4 NanoBiT assay .................................................................................................60
18 Additional strengths and limitations ....................................................................62
19 Future directions .................................................................................................63
20 Conclusion ..........................................................................................................63

References ..................................................................................................................65
Copyright Acknowledgements ..................................................................................76
List of Tables

Table 1 General characteristics of D1-class and D2-class dopamine receptors ...................... 4

Table 2 List of constructs generated ......................................................................................... 28

Table 3 List of other constructs used for cloning and/or experiments ..................................... 29
List of Figures

Figure 1 General mechanisms of GPCR activation and desensitization ........................................ 2
Figure 2 D2R signaling overview ........................................................................................................ 8
Figure 3 D2R signaling to GSK3 via an Akt-βarr2-PP2A complex ................................................... 9
Figure 4 Arrestins origins and classification .......................................................................................... 13
Figure 5 Overview of arrestin structure ................................................................................................. 15
Figure 6 Substrates and functions of the Akt signaling network ......................................................... 18
Figure 7 Molecular mechanisms of Akt regulation ................................................................................ 20
Figure 8 Active pAkt seems to have a better affinity for βarr2 than inactive Akt ......................... 38
Figure 9 βarr2 expression and purification after optimization by Ernst’s lab ................................. 38
Figure 10 Expression test of Akt mutant constructs ............................................................................. 40
Figure 11 Akt-PH domain expression and purification by Ernst’s lab ............................................. 41
Figure 12 Recombinant Akt PH domain and βarr2 show a weak interaction in vitro ................ 42
Figure 13 Schematics of βarr2-derived constructs for co-IP and BRET studies of Akt PH domain and βarr2 interaction ..................................................................................................................... 43
Figure 14 Schematics of some of the Akt constructs for co-IP and BRET studies of Akt PH domain and βarr2 interaction ..................................................................................................................... 45
Figure 15 Akt-PH148 and Myr-ΔPH-Akt1 interact with βarr2-sp1 in co-IPs ............................... 46
Figure 16 Preliminary co-IP verifications of the Akt-PH domain interaction with βarr2 .......... 47
Figure 17 Akt1-PH48, but not Myr-ΔPH-Akt1, interacts with βarr2-sp1 in BRET assays ........ 49
Figure 18 BRET confirmation of the PH domain interaction with βarr2 ........................................ 49

Figure 19 BRET Akt isoforms comparison suggests that Akt2 PH domain also interacts with βarr2
.................................................................................................................................................. 50

Figure 20 TATPEP disrupts the interaction between Akt1-PH148 and βarr2-sp1 in BRET assays
.................................................................................................................................................. 51

Figure 21 Representation of the testing pairs to be used for the NanoBiT assay ...................... 53

Figure 22 Protocol confirmation and orientation test trial for the NanoBiT assay.................... 55
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbr.</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>aa</td>
<td>Amino acid(s)</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylate Cyclase</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein Kinase B or PKB</td>
</tr>
<tr>
<td>Arr</td>
<td>Arrestin</td>
</tr>
<tr>
<td>Arr3</td>
<td>Arrestin 3 (or βarr2)</td>
</tr>
<tr>
<td>β2AR</td>
<td>beta 2 adrenergic receptor</td>
</tr>
<tr>
<td>BP</td>
<td>Bipolar Disorder</td>
</tr>
<tr>
<td>BRET</td>
<td>Bioluminescence Resonance Energy Transfer</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>Co-IP</td>
<td>Co-Immunoprecipitation</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>D2R</td>
<td>Dopamine D2 Receptor</td>
</tr>
<tr>
<td>FOXO</td>
<td>Forkhead Box O</td>
</tr>
<tr>
<td>GIRQ</td>
<td>G Protein-Coupled Inwardly Rectifying Potassium Channel</td>
</tr>
<tr>
<td>GPCR</td>
<td>G Protein-Coupled Receptor</td>
</tr>
<tr>
<td>G proteins</td>
<td>Guanine Nucleotide-Binding Proteins</td>
</tr>
<tr>
<td>GRK</td>
<td>G Protein-Coupled Receptor Kinases</td>
</tr>
<tr>
<td>GSK-3</td>
<td>Glycogen Synthase Kinase 3</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>Li+</td>
<td>Lithium</td>
</tr>
<tr>
<td>LiCl</td>
<td>Lithium Chloride</td>
</tr>
<tr>
<td>mTORC2</td>
<td>Mechanistic Target of Rapamycin Complex 2</td>
</tr>
<tr>
<td>NanoBiT</td>
<td>NanoLuc Binary Technology</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear Export Sequence</td>
</tr>
<tr>
<td>pAkt</td>
<td>Phosphorylated Akt</td>
</tr>
<tr>
<td>PDK1</td>
<td>Phosphoinositide-Dependent Protein Kinase 1</td>
</tr>
<tr>
<td>PH domain</td>
<td>Pleckstrin Homology Domain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-Kinase</td>
</tr>
<tr>
<td>PI3,4P₂</td>
<td>PtdIns-3,4-P₂</td>
</tr>
<tr>
<td>PI4,5P₂</td>
<td>PtdIns-4,5-P₂</td>
</tr>
<tr>
<td>PIC</td>
<td>Protease Inhibitor Cocktail</td>
</tr>
<tr>
<td>PIP₃</td>
<td>PtdIns-3,4,5-P₃</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and Tensin Homologue Deleted on Chromosome 10</td>
</tr>
<tr>
<td>PtdIns</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein Phosphatase 2A</td>
</tr>
<tr>
<td>RGS</td>
<td>Regulators of G protein Signaling</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
</tr>
<tr>
<td>S473</td>
<td>Serine 473</td>
</tr>
<tr>
<td>SHIP</td>
<td>5′-phosphatase SH2-domain-containing inositol 5′-phosphatase</td>
</tr>
<tr>
<td>SZ</td>
<td>Schizophrenia</td>
</tr>
<tr>
<td>T308</td>
<td>Threonine 308</td>
</tr>
<tr>
<td>TAT</td>
<td>Transactivator of Transcription Peptide</td>
</tr>
<tr>
<td>TATPEP</td>
<td>TAT Peptide</td>
</tr>
<tr>
<td>TATSCB</td>
<td>TAT Scrambled</td>
</tr>
<tr>
<td>βArr</td>
<td>Beta-arrestin</td>
</tr>
<tr>
<td>βArr2</td>
<td>Beta-arrestin 2 or arrestin 3</td>
</tr>
</tbody>
</table>
Section 1
Introduction

1 GPCRs

G-protein-coupled receptors (GPCRs), also called 7 transmembrane receptors (7TMRs) due to their highly conserved seven transmembrane alpha helices, form the largest family of membrane receptors in humans with \( \approx 800 \) members, and their importance as therapeutic targets is widely recognized. Indeed, they present the largest family of targets for approved drugs, with about 35% of currently approved drugs in the United States or European Union (Sriram and Insel, 2018; Santos et al., 2017). GPCRs are also intensively studied as major targets of drug discovery (Hauser et al., 2017). In this work, a signaling pathway downstream of a specific GPCR, the dopamine D2 receptor (D2R), will be studied.

1.1 A brief GPCR signaling overview

To understand better the signaling mechanisms downstream of D2Rs, a brief overview of GPCR signaling will prove useful. GPCRs form a very diverse family, with corresponding diverse structures and functions (Fredriksson et al., 2003). However, they generally share certain similarities in their signaling mechanisms (Gurevich and Gurevich, 2019) (Figure 1). First, upon binding by an agonist, GPCRs will undergo structural changes allowing for their activation and binding by G proteins (Dong, Goddard and Abrol, 2017; Latorraca, Venkatakrishnan and Dror, 2017; Ding, Zhao and Watts, 2013). The G proteins will in turn signal to various effectors and this canonical signaling of GPCRs can lead to various effects, depending on the receptor under study, but also on other factors such as the type of G protein subunits involved, the second messengers involved or the effectors subsequently implicated (Beaulieu and Gainetdinov, 2011; Robillard et al., 2000). For example, some classes of \( \alpha \) subunits are involved in the regulation of cAMP production by stimulation or inhibition of adenylate cyclase (AC), resulting in cAMP levels modulation and subsequent alterations in downstream signaling (Neves, Ram and Iyengar, 2002). Activated GPCRs can then be phosphorylated by G protein-coupled receptor kinases (GRKs).
(Ribas et al., 2007). This allows recruitment of arrestin proteins (Claing et al., 2002) which prevents further G protein binding to the receptor, therefore causing desensitisation of the GPCR. This can also lead to mainly two different possible functional outcomes: internalization and trafficking/recycling of the receptor (Tian, Kang and Benovic, 2014), or further signaling where arrestins would serve as a scaffold to recruit more signaling partners (Lefkowitz and Shenoy, 2005; Smith and Rajagopal, 2016; Peterson and Luttrell, 2017). For example, it has been shown that the Raf-MEK-ERK pathway can be activated upon beta 2 adrenergic receptor (β2AR) stimulation in a beta-arrestin (βarr) dependent but G protein independent fashion. Incidentally, this βarr interaction with the ERK signaling pathway has also been noted to occur with activation of other receptors (Shenoy et al., 2006; Tohgo et al., 2002; Eishingdrelo et al., 2015).

Figure 1 General mechanisms of GPCR activation and desensitization

(1) The inactive receptor consists of a closed 7TM helical bundle. (2) Agonist binding stabilizes the active receptor, which couples to a heterotrimeric G protein. (3) The active receptor is also phosphorylated on its C-terminus by a GRK. (4) The phosphorylated, active receptor is bound by arrestin, which blocks further G protein coupling. Depending on the extent and pattern of receptor phosphorylation, arrestin binding can have different functional outcomes. (5) Receptors can be internalized into clathrin-coated pits. (6) Arrestin can induce G protein-independent signaling by scaffolding other signalling proteins. 7TM: 7 transmembrane, GRK: GPCR kinase. Taken from (Scheerer and Sommer, 2017) Figure 1 (a).
1.2 Biased signaling of GPCRs

GPCRs generate their fair part of interest as therapeutic targets (Hauser et al., 2017). In this regard, a relatively recent concept has been gaining attention: biased signaling (Rankovic, Brust and Bohn, 2016). There exists a variety of pleiotropic receptors, that is, receptors linked/coupled to more than one signaling pathway. Biased signaling refers to the idea that some of these signals could be favored at the expense of others (Kenakin, 2019). For example, in GPCRs, biased signaling can often refer to the possibility of activating preferentially either G protein or arrestin signaling (Whalen, Rajagopal and Lefkowitz, 2011). This presents therapeutic interest since different signaling modalities can mediate different effects of a same GPCR. For example, mu and kappa opioid receptors, as well as CB1 receptors, have been actively studied for pain relief effects mediated by G protein signaling. Meanwhile, most side effects associated with drugs targeting these same receptors seem to be mediated by arrestin recruitment. Therefore, using a receptor ligand which is biased for the pathway most recognized for the desired therapeutic effects may be a compelling way to avoid or at least reduce side effects associated with a treatment (Raehal and Bohn, 2014; Brust et al., 2016; Kennedy et al., 2018; Schmid et al., 2017).

In the case of the D2R signaling pathway studied in this work, arrestin-mediated signaling would be involved in undesirable outcomes associated with pathology (see section 2). Therefore, it would seem interesting to selectively antagonize arrestin signaling while trying to avoid affecting G protein signaling, in hopes to avoid undesirable side effects (Komatsu, Fukuchi and Habata, 2019). However, despite offering a strong rationale and a highly attractive potential for therapeutic applications, drug development based on biased signaling still presents several challenges, such as: a need for a better quantification of the bias to minimize system and/or observational bias, a need for more structural and conformational data for biased ligands and, importantly, a need to find a way to bridge the translation gap from in vitro and cell-based assays to in vivo and therapeutic conditions (Kenakin, 2019; Seyedabadi, Ghahremani and Albert, 2019). Moreover, in the case of the D2R signaling pathway studied here (see section 2), potential D2R biased ligands that could differentially affect arrestin or G protein signaling might not necessarily further differentiate between different arrestin-mediated functional outcomes such as internalization/trafficking of receptors as opposed to further arrestin-mediated signaling. In view of these considerations, one alternative to biased signaling would be a more specific targeting of the downstream effectors themselves. This is the approach chosen in this project with targeting of
a protein-protein interaction occurring downstream of D2Rs: the Akt-βarr2 interaction, the importance of which will be described in the next sections.

2 D2R

Dopamine (DA) is a catecholaminergic monoamine neurotransmitter synthesized from the amino acid tyrosine (Hyman, 2005; Grant, 2015). Its action is possible upon activation of the members of a family of 5 dopamine receptors, which are all GPCRs and are named from D1 to D5. Dopamine receptors can generally be divided into D1-class (D1 and D5) and D2-class (D2, D3 and D4). These classes present differences in G protein coupling, adenylate cyclase (AC) and cAMP modulation, cellular localisation and gene structures (see Table 1) (Beaulieu and Gainetdinov, 2011).

**Table 1 General characteristics of D1-class and D2-class dopamine receptors**

A few characteristics outlining differences between D1-class and D2-class dopamine receptors. AC: adenylate cyclase, cAMP: cyclic AMP.

<table>
<thead>
<tr>
<th></th>
<th>D1-class</th>
<th>D2-class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Members</td>
<td>D1 and D5</td>
<td>D2, D3 and D4</td>
</tr>
<tr>
<td>G protein coupling</td>
<td>Gαs, Gαolf</td>
<td>Gαi, Gαo</td>
</tr>
<tr>
<td>Effect on AC and cAMP production</td>
<td>Stimulation</td>
<td>Inhibition</td>
</tr>
<tr>
<td>Expression pre- or post-synaptically</td>
<td>Exclusively postsynaptic</td>
<td>Pre-synaptic (autoreceptors) and post-synaptic</td>
</tr>
<tr>
<td>Gene structure (presence of introns in coding genes)</td>
<td>No introns in genes</td>
<td>Several introns in genes</td>
</tr>
</tbody>
</table>

DA and its receptors are involved in a variety of functions ranging from locomotor activity, reward and reinforcement, learning and memory in the central nervous system (CNS) to olfaction, vision,
hormonal regulation and renal function mediation in periphery. Consequently, DA and DA receptors are also involved in a plethora of pathophysiology, and some of the most recognized cases concern disorders of the CNS such as Parkinson’s disease, addiction, and mental illnesses like bipolar disorder (BP) and schizophrenia (SZ) (Beaulieu and Gainetdinov, 2011). In this thesis, the focus will be on a protein-protein interaction downstream of a D2-class receptor, D2R. Indeed, in the context of D2Rs implication in mental health challenges such as BP and SZ, the Akt-βarr2 interaction will be studied.

2.1 D2R importance in neuropsychiatric disorders

2.1.1 Bipolar disorder and schizophrenia

DA and D2Rs have been implicated in numerous mechanisms and, notably, in several neuropsychiatric disorders, including mental illnesses such as BP and SZ. SZ is a mental illness characterized by positive symptoms (such as psychosis, delusions and hallucinations), negative symptoms (such as amotivation, anhedonia and social withdrawal) and cognitive symptoms (cognitive impairments). Although SZ presents a low prevalence of about 1% in the general population, the illness is highly debilitating and presents a poor prognostic. It has notably been reported that SZ patients have high rates of unemployment and shorter life expectancies (Owen, Sawa and Mortensen, 2016; van Os and Kapur, 2009; Kahn et al., 2015). Meanwhile, BP is a mental illness in which patients experience recurrent episodes of manic, depressive or mixed symptoms. As with SZ, BP presents a relatively low incidence (estimated at about 4% in the United states), but serious consequences: along with elevated suicide risks and life-long effects, BP has been estimated to cause an economic burden of almost $120 billion in the United States in 2009 (McCormick, Murray and McNew, 2015; Miller, 2006; Laursen, Nordentoft and Mortensen, 2014). While both illnesses could be discussed more extensively, it seems enough here to underline that both SZ and BP are serious and debilitating conditions. Moreover, current pharmacological treatments present several limitations and side effects. Antipsychotics are notably recognized for causing metabolic syndrome (Ijaz et al., 2018), among various other undesirable side effects (Tandon, 2011; Siafis et al., 2018; Jeon and Kim, 2017; Werner and Covenas, 2014), while mood stabilizers also present adverse side effects (Bai et al., 2019). Notably, lithium, predominant in BP treatment, has a narrow therapeutic window which necessitates close monitoring and can cause
progressive renal failure and thyroid toxicity (McCormick, Murray and McNew, 2015). More research on SZ and BP seems therefore necessary in hope to improve therapies and outcomes for patients affected by those illnesses.

2.1.2 D2R implication in BP and SZ

Although BP and SZ seem to result from a complex interplay between genes, physiological imbalances and environmental factors (Tandon, Keshavan and Nasrallah, 2008; Scolnick, 2017; Rowland and Marwaha, 2018), both illnesses do present evidence of DA related underlying mechanisms, and this can serve as a basis for further investigations. Indeed, it was demonstrated that most of the clinically effective antipsychotics, drugs used for treatment of SZ and which can also be used for BP, act by antagonism of D2R and, more specifically, by antagonism of a D2R-βarr2 interaction (Masri et al., 2008). This incidentally reinforces what is called the dopamine hypothesis of SZ in demonstrating the importance of DA in SZ (Seeman et al., 2005; Seeman, 2013; Madras, 2013). A role for DA in BP’s etiology has also been suggested (Ashok et al., 2017) and some mood stabilizers used for BP treatment were also shown to interfere with D2R signaling, interestingly in a similar fashion of disruption of D2R-βarr2 signaling (Del’ Guidice and Beaulieu, 2015). Furthermore, it was documented that lithium seems to act by disrupting glycogen synthase kinase 3 (GSK3) signaling occurring downstream of D2Rs via βarr2 (Beaulieu et al., 2004; Beaulieu et al., 2008; Beaulieu and Caron, 2008). In this context, this thesis will take a closer look at a signaling pathway that seems of critical importance for the action of antipsychotics and mood stabilizers, and which seems therefore relevant for BP and SZ. This signaling pathway involves D2R signaling to GSK3 via an Akt-βarr2-PP2A complex and will be further described in the following sections.

2.2 D2R signaling

2.2.1 D2R signaling

D2Rs are mainly expressed in the striatum, nucleus accumbens and olfactory tubercle (Beaulieu and Gainetdinov, 2011). Although D2Rs functions in striatum are recognized, the details of the networks involved for each of these various functions are complex and still a subject under investigation, as reviewed in (Gallo, 2019). It is also worth mentioning that association of D2Rs
with other receptors could be involved in D2Rs regulation and function (Beaulieu and Gainetdinov, 2011; Hasbi, O'Dowd and George, 2010; Ferre et al., 2016). These considerations, among others, likely play important roles in D2Rs functions, but will not be covered in the scope of this thesis. Instead, the focus will be here directed on downstream signaling of D2Rs, more specifically on βarr-mediated signaling. To give a context, an overview of D2R signaling will be presented (see Figure 2).

D2Rs are GPCRs and, upon activation by dopamine, G protein signaling is activated. In the case of D2Rs, it is generally recognized that their coupling with Goi/o results in adenylate cyclase (AC) and cAMP production inhibition, therefore affecting protein kinase A (PKA) signaling. Gβ and Gγ subunits are believed to inhibit L/N-Type calcium channels, while activating G protein-coupled inwardly rectifying potassium channel (GIRK) and phospholipase C (PLC) (Beaulieu and Gainetdinov, 2011). G protein signaling can be negatively regulated by regulators of G protein signaling (RGS), but phosphorylation of active receptors by G protein-coupled receptor kinases (GRKs) also contributes to desensitisation of D2R to G proteins. Additionally, it favors recruitment of arrestins, which can lead to either internalization/trafficking of the receptor or further arrestin-mediated signaling. This latter outcome of arrestin-mediated signaling can be considered as late D2R signaling as it involves more progressive and long-lasting responses. Importantly, this modality of D2R signaling is believed to be important for the action of several antipsychotics and mood stabilizers (Del' Guidice and Beaulieu, 2015; Masri et al., 2008), and will be the focus of this project.
Upon activation by DA (A), D2R can engage G protein signaling, which can be considered as early D2R signaling (B) since this response occurs rapidly and transiently. Indeed, D2Rs can then be desensitized by GRKs, which will additionally favor recruitment of arrestins (C). Arrestins can in turn lead to internalization/trafficking of the receptor (D) or to further βarr-mediated signaling, considered as late D2R signaling in reason of more progressive and long-lasting responses. In this thesis, a closer look will be taken at D2R’s βarr-mediated signaling, highlighted in green.

**Figure 2 D2R signaling overview**

Upon activation by DA (A), D2R can engage G protein signaling, which can be considered as early D2R signaling (B) since this response occurs rapidly and transiently. Indeed, D2Rs can then be desensitized by GRKs, which will additionally favor recruitment of arrestins (C). Arrestins can in turn lead to internalization/trafficking of the receptor (D) or to further βarr-mediated signaling, considered as late D2R signaling in reason of more progressive and long-lasting responses. In this thesis, a closer look will be taken at D2R’s βarr-mediated signaling, highlighted in green.
2.2.2 D2R signaling to GSK3 via an Akt-βarr2-PP2A complex

If the influence of DA and D2Rs in neuropsychiatric disorders is recognized, the details of the underlying mechanisms are still under investigation. However, a specific D2R signaling pathway has been suggested to mediate D2Rs involvement in behaviors related to SZ and BP. This signaling pathway, which is βarr-mediated, can be considered as a late modality of D2R signaling. More specifically, it involves the modulation of GSK3 by D2Rs via an Akt-βarr2-PP2A complex (Figure 3).

Upon DA activation of D2R, βarr2 would form a complex with Akt and PP2A, in which Akt would be dephosphorylated (on T308 residue) by PP2A, and therefore inactivated. Usually, Akt phosphorylation of GSK3 deactivates GSK3 (which is constitutively active). Downstream of D2Rs, deactivation of Akt as the inhibitor of GSK3 would therefore favor GSK3 activity. Interestingly, it seems that most currently clinically effective antipsychotics, and some mood stabilizers as well, would act as antagonists of this βarr2-mediated D2R signaling pathway. Meanwhile lithium, used in treatment of BP, has been shown to inhibit GSK3 directly or indirectly via Akt inhibition.

**Figure 3 D2R signaling to GSK3 via an Akt-βarr2-PP2A complex**

Upon DA activation of D2R, βarr2 would form a complex with Akt and PP2A, in which Akt would be dephosphorylated (on T308 residue) by PP2A, and therefore inactivated. Usually, Akt phosphorylation of GSK3 deactivates GSK3 (which is constitutively active). Downstream of D2Rs, deactivation of Akt as the inhibitor of GSK3 would therefore favor GSK3 activity. Interestingly, it seems that most currently clinically effective antipsychotics, and some mood stabilizers as well, would act as antagonists of this βarr2-mediated D2R signaling pathway. Meanwhile lithium, used in treatment of BP, has been shown to inhibit GSK3 directly or indirectly via Akt inhibition.
A first insight into how D2Rs exert their action was the indication of an important role of βarr2 in behavioral responses induced by dopamine or drugs enhancing dopamine neurotransmission. Indeed, the effects of amphetamine and apomorphine (Beaulieu et al., 2005; Gainetdinov et al., 2004), although not cocaine (Bohn et al., 2003), are reduced in βarr2 knockout (KO) mice, in comparison with wild-type (WT) mice. Additionally, DAT KO mice lacking also βarr2 presented a decrease in novelty-induced hyperlocomotion typically characteristic of DAT KO mice (Beaulieu et al., 2005). These findings, combined with observations of the importance of D2-βarr2 interactions for the action of several clinically effective antipsychotics and mood stabilizers (Masri et al., 2008; Del' Guidice and Beaulieu, 2015), indicate that βarr2 plays a critical role in D2R signaling.

Further studies revealed that DAT KO mice present a reduced phosphorylation/activity of Akt in comparison with WT mice (Beaulieu et al., 2004; Beaulieu et al., 2006). A reduction in active/phosphorylated Akt levels following apomorphine or amphetamine treatment of WT mice also confirms Akt regulation by DA receptors (Beaulieu et al., 2005). Furthermore, this modulation of Akt by D2R is apparently mediated by βarr2, but not affected by cAMP. Indeed, DAT KO mice lacking βarr2 did not present a decrease in Akt phosphorylation levels (Beaulieu et al., 2005). Amphetamine and apomorphine similarly did not reduce phosphorylated Akt levels in βarr2 KO mice (Beaulieu et al., 2005). Since modulation of cAMP levels in the striatum of mice did not affect Akt (Beaulieu et al., 2005), it seems that D2Rs modulate Akt dephosphorylation/deactivation in a βarr2 dependent but cAMP independent manner.

Akt is a serine/threonine kinase involved in multiple physiological processes (see section 4) that has been shown to inhibit GSK3 in response to a variety of growth factors or hormones such as insulin, insulin-like growth factors and BDNF. GSK3 is also a serine/threonine kinase, discovered for its role in glycogen biosynthesis (Embi, Rylatt and Cohen, 1980; Frame and Cohen, 2001), and represented by two closely related isoforms, GSK3α and GSKβ, which are expressed ubiquitously in mammalian tissues. GSK3 is constitutively active and is inactivated when phosphorylated by Akt (on S21 for GSK3α and S9 for GSKβ) (Hermida, Dinesh Kumar and Leslie, 2017; Chen and Russo-Neustadt, 2005; Frame and Cohen, 2001; Cross et al., 1995). GSK3 is believed to be involved in a plethora of processes, ranging from glycogen synthesis to embryonic development as well as synaptic plasticity and regulation of circadian behavior via D2R (Hermida, Dinesh Kumar and Leslie, 2017; Woodgett, 1990; Beurel, Grieco and Jope, 2015; Beaulieu and
Most importantly here, GSK3 was shown to be affected by a wide range of antipsychotics (Emamian et al., 2004; Li et al., 2007; Alimohamad et al., 2005). Additionally, although lithium’s mechanism of action is still not completely understood (Freland and Beaulieu, 2012; Beaulieu and Caron, 2008), it has been reported that lithium can directly inhibit GSK3 (Klein and Melton, 1996; Sun et al., 2011; Ryves and Harwood, 2001). Lithium has also been documented to be able to inhibit GSK3 indirectly via Akt activation (Pan et al., 2011; Beaulieu et al., 2004).

In this context, it seems that Akt and GSK3 are both regulated downstream of D2Rs via βarr2. Indeed, reduced Akt phosphorylation/activation in DAT KO mice was associated with decreased GSK3 phosphorylation and increased GSK3 activity (for both GSK3α and GSK3β) (Beaulieu et al., 2004). Conversely, still in DAT KO mice striatum, use of the D2/D3 antagonist raclopride or dopamine depletion by αMPT both presented increased Akt and GSK3 phosphorylation (Beaulieu et al., 2004). Meanwhile, SCH23390, a D1-class DA receptor inhibitor, did not affect either Akt or GSK3, further pointing towards a specificity of regulation of Akt and GSK3 by D2Rs (Beaulieu et al., 2004). Additonally, in C57BL6 mice striatum, amphetamine inhibited Akt and favored GSK3 activity (Beaulieu et al., 2004), while haloperidol in WT mice increased Akt phosphorylation and inhibited GSK3 activity (Emamian et al., 2004). Accordingly, in WT mice cortex and striatum, chronic lithium, lamotrigine and valproate were associated with increased Akt and GSK3β ploshorylation (Del’ Guidice and Beaulieu, 2015). These increases did not occur for the same treatments administered to D2R-KO mice or βarr2 KO mice (Del’ Guidice and Beaulieu, 2015), underlining the importance of both D2R and βarr2 in the regulation of Akt and GSK3. Finally, since cAMP modulation did not affect either Akt or GSK3 (Beaulieu et al., 2004), D2R modulation of these two kinases seems independent of cAMP.

Indeed, it seems that D2R regulation of Akt and GSK3 happens via βarr2, more specifically via formation of an Akt-βarr2-PP2A complex. βarr2 KO mice, which showed reduced DA responsiveness, did not show reduction in DARPP-32 activation following amphetamine treatment, indicating that the blunted DA responsiveness might be independent of cAMP and G protein signaling pathways (Beaulieu et al., 2005). However, the inhibiton/dephosphorylation of Akt after treatment with amphetamine and apomorphine was prevented in βarr2 KO mice, and also in DAT KO mice lacking βarr2 (Beaulieu et al., 2005). Investigations on how D2R might promote Akt dephosphorylation then ruled out ERK related mechanisms or PI3K impairments (Beaulieu et al., 2005) and identified a role of protein phosphatase 2A (PP2A). Indeed, treatment of DAT KO
mice with okadaic acid (PP1/PP2A inhibitor) or fostriecin (PP2A inhibitor), both showed increased pAkt levels, indicating that inhibiton of PP2A prevented DA related Akt dephosphorylation (Beaulieu et al., 2005). Additionally, fostriecin specifically prevented dephosphorylation of the T308 residue of Akt, both in DAT KO mice and in WT mice treated with amphetamine, which implies that PP2A would dephosphorylate Akt on its T308 residue (Beaulieu et al., 2005). Confirmation of βarr2 specific interaction with Akt, GSK3 and PP2A was then obtained in vitro (Beaulieu et al., 2005) while it was shown that Akt and PP2A interaction seemed dependent of βarr2 in mouse striatum (Beaulieu et al., 2005). Formation of an Akt- βarr2-PP2A complex also seemed to be regulated by DA, as explored with WT mice treated with amphetamine (Beaulieu et al., 2005). Finally, it was demonstrated that an Akt- βarr2-PP2A complex was important for lithium treatment, and that lithium probably disrupts formation of this complex (Beaulieu et al., 2008).

To summarize, D2Rs implication in the effects of various antipsychotics and mood stabilizers seems to happen by modulation of GSK3 via an Akt-βarr2-PP2A complex. Starting from the observation that a D2R-βarr2 interaction seems crucial in the action of antipsychotics and mood stabilizers, it was then discovered that βarr2 interacts with Akt and PP2A to form a complex, which results in dephosphorylation of Akt’s T308 residue by PP2A. Since active Akt would normally inhibit GSK3, this inhibition of Akt allows disinhibition of GSK3 activity and subsequent behavioral changes. In this project, the intention was to further investigate this signaling pathway and more specifically the Akt-βarr2-PP2A complex by studying the interaction between two of the proteins involved in the formation of this complex, namely the proteins Akt and βarr2. The following sections will take a closer look at each of these proteins.
3  Arrestins and βarr2

3.1  General roles and functions of arrestins

Arrestins seem to originate from common ancestors found in prokaryotes and, more specifically, an Archaea arrestin-like protein family (sporulation stage 0, protein M (SpoOM) family) appears to have diverged into two main families of eukaryotic arrestin-like proteins: α-arrestins and visual/β-arrestins. More details on the origins and taxonomy of arrestins can be found in (Peterson and Luttrell, 2017), but a focus will be given here to βarr2. The latter is a member of the visual/β-arrestins, which distinguish themselves from other arrestin-like proteins by their capacity to interact with GPCRs. This family includes 4 members and can be segregated into visual arrestins (Arr1 (Arr1), Arr4 (Arr4)), and nonvisual arrestins or β-arrestins (Arr2/β-arrestin 1 (Arr2/βarr1), Arr3/β-arrestin 2 (Arr3/βarr2)) (Peterson and Luttrell, 2017) (see Figure 4).

Figure 4  Arrestins origins and classification

Eukaryotic arrestins originate from a common prokaryote ancestor which diverged into two main eukaryotic families of arrestin-like proteins: α-arrestins and visual/β-arrestins. Visual/β-arrestins distinguish themselves from other arrestin-like proteins by their capacity to interact with GPCRs. They can be further classified as visual arrestins (Arr1 and Arr4) or nonvisual/β-arrestins (Arr2/βarr1 and Arr3/βarr2). Further details concerning arrestins taxonomy or α-arrestins can be found in (Peterson and Luttrell, 2017). The protein studied in this project, βarr2, is highlighted in green.
Visual arrestins are mostly expressed in visual sensory tissue such as the retina and are primarily involved in rhodopsin desensitisation. Conversely, nonvisual/βarr are more ubiquitously expressed and are believed to be involved in regulating hundreds of different GPCRS (Peterson and Luttrell, 2017; Gurevich et al., 1995). Indeed, arrestins can act as scaffold proteins in a variety of processes: though lacking themselves intrinsic catalytic activity, they can favor signaling between different components of a same cascade, reduce crosstalk between different cascades and help to target effectors to specific subcellular locations (Peterson and Luttrell, 2017). Arrestins have also been noted to exist in different intracellular functional pools: there can be cytosolic, microtubule-bound, GPCR-bound and nuclear arrestins. It has been observed that each pool could serve different functions, with some arrestin cargos having better affinity for one or another intracellular location, which might be due to variations in the conformation of arrestins in each pool. For example, Ca²⁺-calmodulin, JNK3 and Mdm2 exhibit higher affinity for cytosolic arrestin, while cargos such as ERK 1/2, c-Raf-1, clathrin and AP-2 seem to prefer GPCR-bound arrestins. In the case of βarr2, the presence of a nuclear export sequence (NES) limits it to cytosolic, microtubule-bound and GPCR-bound pools (Peterson and Luttrell, 2017). Arrestin signaling downstream of D2Rs as studied in this work seems to correspond to βarr2 as a GPCR-activated scaffold.

As previously mentioned (section 1) arrestins interacting with GPCRs are recognized to contribute to desensitisation, internalization and trafficking of receptors, leading to their degradation or recycling. However, it has also been demonstrated that they can mediate some late receptor signaling as well, as was mentioned about the Raf-MEK-ERK signaling pathway in section 1. Arrestins are therefore believed to be involved in numerous and diverse processes, ranging from negative regulation of G proteins to modulation of immune response and developmental regulation as well as cell growth and proliferation, cell survival and apoptosis, and cell migration and chemotaxis. A more extensive review of the various cargos, pathways and subsequent functional outcomes in which arrestin signaling can be involved can be found in (Peterson and Luttrell, 2017). In the central nervous system, βarr2 has been implicated in learning and memory via plasticity modulation (Eng et al., 2016) and, of interest for this work, seems essential to modulate dopaminergic neurotransmission related notably to locomotor activity and neural reward mechanisms. Notably, as previously described, D2R in the striatum appears to modulate GSK3 in a βarr2-dependent manner via formation of an Akt-βarr2-PP2A complex (see section 2.2.2).
3.2 Arrestin structure and activation

Although structure variations obviously occur between different arrestin isoforms or between different species, some important features are common to most arrestins structures, and some recurrent structural changes have been recognized to characterise arrestin activation. A few points will be mentioned here, while a more thorough review can be found in (Scheerer and Sommer, 2017) or (Peterson and Luttrell, 2017).

The arrestin family is defined by a conserved fold of 20 β strands which form two crescent-shaped β sandwiches, themselves placed side by side and constituting an N-domain and a C-domain. The central part of the protein forms a central crest or hinge domain, which includes the finger loop/motif II, considered a key receptor-binding element, and two elements implicated in stabilizing basal-state inactive arrestin, the middle loop and the C-loop. A network of buried charged residues at the interface between the N-domain and the C-domain helps to stabilize this same interface and has been designated as polar core. Finally, a C-terminal tail (C-tail), anchored to the N-domain in arrestin basal state, also contribute in stabilizing the basal structure (Scheerer and Sommer, 2017).

**Figure 5 Overview of arrestin structure**

Overview of arrestin structure, here demonstrated on a basal state inactive bovine arrestin 1 (PDB entry 1CF1, molecule A). One can note the N-domain and C-domain basket-like structures, which are joining in the center at the central crest and polar core. The C-tail is here anchored on the N-domain as this arrestin is in basal state. Taken from (Scheerer and Sommer, 2017).
Arrestins in their basal state are considered inactive and mostly unable to bind activated receptors. Their interaction with a receptor seems to rely on detecting a receptor activation and phosphorylation via their phosphate and activation sensors (Peterson and Luttrell, 2017; Chen et al., 2017). Upon activation, arrestins undergo several structural modifications. Indeed, it seems that the C-tail is freed upon interaction with the phosphorylated receptor, allowing access to residues in the N-domain and leading to further conformational changes such as a loosening of the central crest and breakage of the polar core. This in turn allows reinforced binding of the receptor. Further conformational changes then occur, notably an interdomain rotation, considered as one of the hallmarks of activated arrestin (Scheerer and Sommer, 2017; Peterson and Luttrell, 2017). Such activation-related conformational changes seem to apply to βarr2, at least as characterised when activated in a receptor-independent fashion by binding of IP6 to the phosphate sensor (Chen et al., 2017).

4 Akt

4.1 General roles and functions of Akt

Akt is a serine threonine kinase which was discovered more than 25 years ago and presents a rich, complex and diverse interactome. Following work from (Staal, 1987) which identified the v-Akt oncogene from AKT8 retrovirus, the cellular homolog of v-Akt was cloned and characterized in 1991. This happened to be done independently by three different laboratories, each ending up naming the protein differently in regard of the methods they used to characterize it: c-Akt, RAC or PKB (Bellacosa et al., 1991; Jones et al., 1991; Coffer and Woodgett, 1991). Since then, it has been established that three AKT/PKB isoforms exist in mammalian genomes: Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBγ) (Manning and Toker, 2017). Akt is ubiquitously expressed: pretty much all tissues seem to express one or more Akt isoform. However, interestingly, while Akt1 seems to be the most widely expressed isoform, Akt2 appears to be enriched in insulin-responsive metabolic tissues, while Akt3 would be enriched in the brain (Gonzalez and McGraw, 2009; Manning and Toker, 2017). This suggests that, although Akt isoforms present redundant functions in many cases, they can also showcase some isoform-specific functions depending on different tissues and
contexts (Dummler and Hemmings, 2007; Levenga et al., 2017). Additionally, it is generally accepted that cytoplasmic inactive Akt is recruited at the plasma membrane by phosphoinositides, leading to its activation as will be further discussed in section 4.2. However, it is interesting to note that other subcellular locations of Akt, such as various endomembranes, the mitochondria or the nucleus, have been documented, opening the discussion on the role of Akt localisation for its activation and activity, and on the possibility of isoform-specific subcellular locations (Lucic et al., 2018; Sugiyama, Fairn and Antonescu, 2019; Santi and Lee, 2010).

As can be seen in Figure 6, Akt can be involved in multiple processes and cellular functions, and an intricate network of regulation can emerge when taking a closer look (Manning and Toker, 2017). Still, a few key points can be noted. First, Akt is well established as being activated by growth factors and as a downstream effector of phosphoinositide 3-kinase (PI3K), a lipid kinase itself involved in mechanisms such as insulin response, cellular growth and metabolism, inflammation and immunity, warranting its involvement notably in cancer and diabetes (Cantley, 2002; Vanhaesebroeck, Whitehead and Pineiro, 2016; Fruman et al., 2017; Engelman, Luo and Cantley, 2006). Furthermore, although downstream effectors of Akt are abundant, three main signaling nodes can be recognized in FOXO, GSK3 and mTORC1, as described more in details in (Manning and Toker, 2017). Likely, most of the effects of PI3K are mediated by Akt, the latter being involved, through its multiple substrates, in various important cellular functions, which include survival, proliferation, metabolism and growth. This leads to Akt playing a role in several pathophysiologicals: overgrowth syndromes, cancer, abnormalities in endothelial cells, angiogenesis and vascular biology, insulin response and systemic metabolism dysfunctions, immunity and autoimmune diseases and, of interest for this work, neuropsychiatric disorders (Manning and Toker, 2017).

Indeed, although a great part of Akt research concerns its important role in cancer, Akt seems to be also implicated in neuronal survival, growth, polarity, synaptic plasticity and circuitry (Manning and Toker, 2017), therefore linking it to several neuropsychiatric disorders. Akt has for example been implicated in autism spectrum disorders and intellectual disabilities (Onore et al., 2017; Borrie et al., 2017; Lipton and Sahin, 2014) or in Alzheimer’s or other neurodegenerative diseases (Yi et al., 2018; Heras-Sandoval et al., 2014). However, it is Akt’s involvement in SZ and in mood disorders such as BP and depression, that retains attention here (Matsuda et al., 2019). Notably, it seems that decreased Akt signaling, and concomitant increased GSK3 signaling, could underlie
such mental illnesses (Beaulieu, 2012; Zheng et al., 2012; Kaidanovich-Beilin and Woodgett, 2011). This seems in accordance with the relevance for BP and SZ of D2R regulation of GSK3 via formation of an Akt-βarr2-PP2A complex (Beaulieu, Gainetdinov and Caron, 2009; Del' Guidice and Beaulieu, 2015; Beaulieu et al., 2005).

Figure 6 Substrates and functions of the Akt signaling network
Akt phosphorylates downstream substrates that are involved in the regulation of diverse cellular functions, including multifunctional substrates. A partial list of known substrates is shown. P indicates phosphorylation, with red and green denoting inhibitory and activating regulation, respectively. Taken from (Manning and Toker, 2017).
4.2 Akt Structure and Activation

All three Akt isoforms belong to the AGC kinase family and share a common domain organisation. Starting in N-terminal with a pleckstrin homology (PH) domain, linked to a central catalytic kinase domain, Akt is then completed by a C-terminal regulatory domain (Figure 7B) (Hanada, Feng and Hemmings, 2004).

As previously mentioned, classical activation of Akt occurs downstream of PI3K (Figure 7A). Indeed, it seems that stimulation of some receptors tyrosine kinase (RTKs) or GPCRs can lead to activation of class I PI3K, the latter mostly phosphorylating phosphatidylinositol (PtdIns)-4,5-P₂ (PI4,5P₂), forming PtdIns-3,4,5-P₃ (PIP₃) (Vanhaesebroeck et al., 2010). PIP₃ can then recruit Akt to the plasma membrane via binding to its PH domain, which will later lead to Akt activation (Frech et al., 1997; James et al., 1996). Conversely, PIP₃ can be dephosphorylated back to PI4,5P₂ by the tumor suppressor phosphatase and tensin homologue deleted on chromosome 10 (PTEN), therefore interrupting its signalling (Vanhaesebroeck et al., 2010). PIP₃ can also be converted in PtdIns-3,4-P₂ (PI3,4P₂) by 5’-phosphatase SH2-domain-containing inositol 5’-phosphatase (SHIP) and interestingly PI3,4P₂ also can bind Akt PH domain (Franke et al., 1997; Klippel et al., 1997), which might be related to endomembrane pools of active Akt (Manning and Toker, 2017). PI3,4P₂ can also be converted in PI3P by inositol polyphosphate-4-phosphatase, isoform B (INPP4B) (Vanhaesebroeck et al., 2010).

The PH domain of Akt is considered self-inhibitory when Akt is inactive, however upon recruitment by PIP₃ conformational changes occur, resulting in an opening of the structure, releasing the PH domain from its inhibitory interaction with the catalytic kinase domain (Calleja et al., 2007; Calleja et al., 2009). This in turn allows two major phosphorylation events on Akt: phosphorylation of the T308 residue by the phosphoinositide-dependent protein kinase 1 (PDK1) and phosphorylation of the S473 residue by the mechanistic target of rapamycin (mTOR) complex 2 (mTORC2) (Alessi et al., 1996). Of note, while T308 phosphorylation is considered necessary for Akt activity, S473 phosphorylation would play more of a stabilizing role, therefore allowing maximal Akt activation (Alessi et al., 1996; Yang et al., 2002; Manning and Toker, 2017). Additionally, numerous other post-translational modifications of Akt can occur, as described in (Manning and Toker, 2017). For example, recent findings indicate a role for phosphorylation of S477 and T479 in differential activation mechanisms (Chu et al., 2018). Akt inactivation can be
achieved by dephosphorylation of T308 by PP2A and dephosphorylation of S473 by PH domain leucine-rich repeat protein phosphatase (PHLPP) (Manning and Toker, 2017).

Figure 7 Molecular mechanisms of Akt regulation

(A) Stimulation of RTKs or GPCRs leads to activation of PI3K, leading to PIP₃ production at the plasma membrane. Cytosolic inactive Akt is recruited to the membrane and engages PIP₃ through PH domain binding. This leads to phosphorylation of T308 and S473 by PDK1 and mTORC2, respectively, resulting in full activation. Signal termination is achieved by the PIP₃ phosphatase PTEN and the PP2A and PHLPP protein phosphatases. A separate endomembrane pool of active Akt likely exists that is activated through engagement of PI3,4P₂ through the action of the SHIP phosphatase and terminated by INPP4B. (B) The modular structure of Akt1, with position of post-translational modifications color coded for phosphorylation (pSer/pThr/pTyr), acetylation (Lys-Ac), ubiquitylation (Lys-Ub), methylation (Lys-Me), hydroxylation (Pro-OH), glycosylation (O-GlcNac) and SUMOylation (Lys-SUMO). For more details, refer to (Manning and Toker, 2017). Figure taken from (Manning and Toker, 2017).
5 Preliminary results concerning the Akt-βarr2 Interaction

Previous members of the Beaulieu lab have made tremendous work towards a better understanding of the Akt-βarr2 interaction. This work, although not yet published at the moment of writing this thesis, was an essential basis for this project and will be briefly summarized here.

5.1 Active pAkt has a better affinity for βarr2 than inactive Akt

Pulldown assays were performed with recombinant βarr2 (see construct 27) and commercially available non-active Akt and active Akt proteins (Millipore Ltd, 14-270 and 14-276). The results indicated that active pAkt had a better affinity for βarr2 than the inactive Akt. This is in accordance with the concept of dephosphorylation of Akt by PP2A in the Akt-βarr2-PP2A complex. According to this idea, the active pAkt would have an affinity for βarr2 which would allow formation of that complex but, upon dephosphorylation (on T308) by PP2A, Akt would get deactivated and therefore might lose affinity and fall off more easily from the complex.

5.2 Identification of two putative interfaces of interaction on βarr2

Various truncated mutants of βarr2 were generated, and pulldowns of these mutants against commercial pAkt helped to narrow down two plausible regions of interaction, hereafter named as interfaces A and B. Interface A being located at amino acids (aa) 100-110, it appears to be on an α-helix structure. Interface B was identified as aa 150-160, which lands on what seems to be an unstructured loop. In order to further study these putative interaction interfaces, interface B was first selected to generate a TAT (Transactivator of Transcription) peptide (TATPEP) corresponding to its aa sequence. The TAT sequence is intended to enhance cell permeability of the peptide (Bolhassani, Jafarzade and Mardani, 2017; Kim, Moodley and Liu, 2015; Zhang and Wang, 2012; van den Berg and Dowdy, 2011). Pulldown assays later demonstrated that this TATPEP could effectively compete the interaction, further confirming involvement of the interface B in the Akt-βarr2 interaction.
5.3 Confirmation of specificity for βarr2

Additional pulldown assays were run between commercially available Akt and pAkt against different variants of βarr and it was observed that βarr1 interacted less with pAkt than βarr2. βarr2 was then mutated to mimic βarr1 on the interface B: this mutant was termed ENLI, in reference to the corresponding aa of βarr1 involved. ENLI also interacted less with pAkt than βarr2 in pulldown assays. Finally, since all these experiments were made with recombinant rat arrestins, a recombinant human βarr2 was tested in pulldowns as well, and also showed similar results. Taken all together, these results showed pAkt specifically interacts with βarr2, and that the previously identified interface B seems involved in that interaction. Moreover, this seemed generalizable to human βarr2 as well.

5.4 TATPEP does not affect other aspects of the D2R-GSK3β signaling pathway

In order to further confirm the specificity of the effect of the TATPEP on the Akt-βarr2 interaction, several other experiments were conducted. Briefly, it was observed that systemic injection of TATPEP, but not TATSCB (a peptide containing the same aa in a scrambled order), transiently elevated pT308-Akt and pS9-GSK3β levels in the mouse striatum, consistent with the putative disruption of the Akt-βarr2 interaction. TATPEP did not seem to affect either striatal DA levels, βarr (1 or 2) recruitment to D2R, D2R internalisation or βarr2 recruitment of AP2 to D2R. D1R- and D2R-induced changes in cAMP production, as well as G protein- and βarr-mediated ERK1/2 phosphorylation downstream of At1a receptor were also unaltered. TATPEP did not seem to induce proliferation in N2A cells. Finally, amphetamine- and novelty-induced locomotion in mice suggested that the TATPEP could mimic some behavioral effects of lithium, in a βarr2-dependent manner. Further explanations about these experiments will likely be available in future publications from our group. These findings indicate that TATPEP interferes with the Akt-βarr2 interaction in a specific manner.
6 Akt-βarr2 Interaction Project

6.1 Project rationale

As previously noted, modulation of GSK3 by D2R via an Akt-βarr2-PP2A complex seems to play a major role in BP and even SZ related behaviors. Current antipsychotics and mood stabilizers have been demonstrated to affect this signaling pathway, but their efficiency is not always optimal and they often present considerable undesirable side effects. In that perspective, identifying novel approaches targeting the same mechanisms while attempting to avoid side effects seems desirable. Development of βarr2-biased ligands of D2R is an attractive approach under study, but might not target specifically the βarr2-mediated signaling since it may also affect βarr2-mediated trafficking of the receptor. Considering the different modalities of signaling associated with D2R, it seems interesting here to target more precisely the Akt-βarr2-PP2A complex downstream of D2R. This should avoid affecting not only G protein signaling but also arrestin-mediated receptor desensitization and internalization. However, in order to target the Akt-βarr2-PP2A complex, getting a better understanding of how the components of this complex interact seems essential. Therefore, this project focused on elucidating the interaction between two major proteins involved in this complex: Akt and βarr2. Considering the preliminary data detailed above, a site of interaction on βarr2 was identified. However, the corresponding site of interaction on the Akt side remains to be clarified if the Akt-βarr2 interaction is to be targeted for eventual drug development, and this aspect was investigated in this work.

6.2 Research Hypotheses

6.2.1 Phosphorylation sites of Akt can lead to the interaction site of Akt with βarr2

Since preliminary data from the Beaulieu lab indicates that active pAkt interacts more than inactive Akt with Barr2, it was further hypothesized that different phosphorylation sites, namely the residues T308 and S473, recognized for their role in Akt activation, might affect differentially Akt’s affinity for βarr2. Considering that PP2A has been shown to dephosphorylate T308, and that T308 phosphorylation is deemed essential for Akt activation, phosphorylation at this residue seems relevant to study. Additionally, phosphorylation of the S473 residue was also noted to play an
important role in Akt activation. Investigating if there might be any differential effect of these two phosphorylation sites on the Akt-βarr2 interaction might contribute to guide future efforts to identify an interaction site on Akt for βarr2. Additionally, this might lead to the development of interesting tools to study the dynamics of the interaction.

6.2.2 The PH domain of Akt may present an interaction site of Akt with βarr2

The PH domain of Akt can be deleted for protein expression optimisation (Klein et al., 2005). However, its binding to PIP₃ and subsequent Akt activation induce structural changes in the PH domain. This opens the possibility that Akt’s interaction with βarr2 could happen on the PH domain as well as on the catalytic kinase domain. Recombinant Akt expression is planned in order to further study the Akt-βarr2 interaction, notably for study of the hypothesis 6.2.1, and truncating the PH domain is considered as a means of optimising expression. However, before pursuing experiments with truncated constructs, it seems relevant to verify if the PH domain of Akt might interact with βarr2.

6.3 Specific Objectives

6.3.1 Investigation of the effect of different phosphorylation sites of Akt on its interaction with βarr2

6.3.1.1 Cloning of pseudo-phosphorylated mutants of Akt

In order to study the role of different phosphorylation sites of Akt in its interaction with βarr2, cloning of Akt mutants pseudo-phosphorylated on two key phosphorylation sites deemed important for Akt activation is planned. Therefore T308E, S473E and double mutant (T308E and S473E) Akt constructs will be generated in view of production of recombinant Akt proteins that aim to mimic active phosphorylated Akt. Additionally, a version of each of these mutants with a truncated PH domain will also be made in view of possible optimisation of protein expression.

6.3.1.2 Expression and purification of recombinant proteins

In collaboration with Dr Ernst’s lab, recombinant Akt and βarr constructs will be expressed and purified.
6.3.1.3 In vitro testing of purified proteins by pulldown assays

The purified recombinant proteins will serve to replicate previous data from the Beaulieu lab and to study the effects of different Akt phosphorylation sites on its interaction with βarr2.

6.3.2 Investigation of a possible interaction site on the Akt PH domain

6.3.2.1 Cloning of constructs for BRET and Co-IPs

Existing and new constructs of Akt and βarr2 will be designed and cloned to be used in BRET and co-IPs.

6.3.2.2 Testing of the constructs in Co-IPs

Co-immunoprecipitation assays (Co-IPs) will be used as a first verification of the interaction between various constructs previously generated. This method can confirm interaction between proteins, but as a limitation can be difficult to quantify precisely. This method and BRET assays will therefore be used in a complementary manner, as BRET might provide a more quantitative insight but remains a proximity assay.

6.3.2.3 Testing of the constructs in BRET

Bioluminescence resonance energy transfer (BRET) will be used to verify the interaction between various constructs of Akt and βarr2 previously generated. As mentioned above, BRET and Co-IPs approaches aim to complement each other. Since BRET is a proximity assay, use of co-IPs will confirm the interaction in a more direct manner.

6.3.3 Development of a split-luciferase NanoBiT assay for future screening of compounds

6.3.3.1 Cloning of NanoBiT constructs

In view of scaling up for possible screening of libraries of small compounds, for possible drug development, constructs will be generated for a split-luciferase assay based on the NanoBiT (NanoLuc Binary Technology) system (Promega).
6.3.3.2 Testing of the protocol

The protocol for the NanoBiT split-luciferase assay will be established and troubleshooted in our laboratory.

6.3.3.3 Orientation test

Various test pairs will be used to determine which tag orientation on each protein gives a better signal to noise ratio and therefore would be optimal for further screenings.
Section 2
Materials and Methods

7 Cloning

7.1 Cloning Methods

Site-directed mutagenesis was designed and performed according to guidelines from Thermo Scientific Phusion Site-Directed Mutagenesis Kit (Pub. No. MAN0013377, Rev. B. 00) (#F-541) (Thermo Fisher Scientific Inc.) for constructs 2-8 inclusively. For constructs 9-13 inclusively mutagenesis was designed and performed following recommendations from NEBaseChanger (http://nebasechanger.neb.com/) (New England BioLabs Inc.). The change of mutagenesis method was due to a more efficient and successful process with the NEBaseChanger recommendations. Constructs 1 and 14-20 inclusively were designed and generated following Gibson Assembly guidelines from NEBuilder, versions 1 and 2 (http://nebuilder.neb.com/#/) (New England BioLabs Inc.). Note that all βarr2-derived constructs for BRET and Co-IPs were tagged in N–term with the donor Rluc8. Conversely, all Akt-derived BRET constructs were tagged in C-term with the acceptor GFP. All clonings were verified by sequencing via The Centre for Applied Genomics (TCAG).

7.2 List of constructs

The following tables present a list of all constructs used in the scope of this MSc thesis. Maps of the constructs are available upon request.
Table 2 List of constructs generated

List of constructs generated, with specifications and alternative names in parentheses. All constructs are ampicillin/carbenicillin resistant. 2XM: double mutant (T308E and S473E), ΔPH: deleted PH domain (aa1-111).

<table>
<thead>
<tr>
<th>Constructs for in vitro studies of pseudo-phosphorylated Akt mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>All constructs in pGEX4T-1 vector and based on mouse Akt1 (see construct 23). All constructs were tagged with Twin-Strep-tag (Schmidt et al., 2013) in N-terminal and 6His tag in C-terminal.</td>
</tr>
<tr>
<td>1 - pGEX-Akt1 (AktX)</td>
</tr>
<tr>
<td>3 - pGEX-Akt1-T308E (T308E)</td>
</tr>
<tr>
<td>5 - pGEX-Akt1-S473E (S473E)</td>
</tr>
<tr>
<td>7 - pGEX-Akt1-2XM (2XM)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Constructs for BRET and Co-IP studies of Akt PH domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>All constructs in pcDNA3.1 (see construct 21) and based on either a bovine βarr2 transcript variant (see construct 31), human Akt1 (GenBank M63167.1), human Akt2 (see construct 25) or human Akt3 (see construct 26)</td>
</tr>
<tr>
<td>9 - Rluc8-βarr2 (without sp1)</td>
</tr>
<tr>
<td>11- Rluc8-ENLI (ENLI)</td>
</tr>
<tr>
<td>13 - Rluc8-βarr2aa1-175 (βarr2-175)</td>
</tr>
<tr>
<td>15 - Akt2-PH117-GFP (Akt2-PH117)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Constructs for NanoBiT split-luciferase assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constructs prepared from NanoBiT MCS Starter System (See constructs 37-40 inclusively) (Promega, N2014)</td>
</tr>
<tr>
<td>17 - LgBiT-βarr2-sp1 (LB)</td>
</tr>
<tr>
<td>19 - Akt1-PH148-LgBiT (AL)</td>
</tr>
<tr>
<td>Constructs used primarily for cloning</td>
</tr>
<tr>
<td>--------------------------------------</td>
</tr>
<tr>
<td>21 - pcDNA3.1 (empty vector)</td>
</tr>
<tr>
<td>22 - pGEX4T-1 (empty vector)</td>
</tr>
<tr>
<td>23 - pcDNA-Akt1 (based on mouse Akt1: GenBank X65687.1)</td>
</tr>
<tr>
<td>24 - 1017 pcDNA3 Myr Akt1 deltaPH (Addgene #9009)**</td>
</tr>
<tr>
<td>25 - pmCherry-Akt2(WT) (Addgene #86623) (hAkt2)**</td>
</tr>
<tr>
<td>26 - 1236 pcDNA3 Myr HA Akt3 (Addgene #9017) (hAkt3)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Constructs used primarily for in vitro experiments</th>
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</tr>
</thead>
<tbody>
<tr>
<td>All constructs in pGEX4T-1</td>
<td></td>
</tr>
<tr>
<td>27 – GST-βarr2 (based on rat βarr2: GenBank BC087578.1)</td>
<td></td>
</tr>
<tr>
<td>28 - GST</td>
<td></td>
</tr>
<tr>
<td>29 - βarr2 (Arr3 untagged) (based on a modified bovine βarr2 transcript variant (see construct 31)) (in a modified pTrcHis B vector, with the His tag removed)*</td>
<td></td>
</tr>
<tr>
<td>30 - GST-Akt1-PH123 (based on mouse Akt (see construct 23) (Akt1-PH123)*</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Constructs used primarily for BRET and Co-IPs</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>31 - Rluc8-arr3-sp1 (based on a modified βarr2 transcript variant: NCBI Reference Sequence: XM_005220181.4, modifications as K53R and V263M) (βArr2-sp1)</td>
<td></td>
</tr>
<tr>
<td>32 - pcDNA3-AKT-PH-GFP (Addgene #18836) (Akt1-PH148)</td>
<td></td>
</tr>
<tr>
<td>33 - pcDNA-GFP (GFP)</td>
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<table>
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<tr>
<th>Constructs from NanoBiT PPI MCS Starter System (Promega, N2014)</th>
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</tr>
</thead>
<tbody>
<tr>
<td>34 - LgBiT-PRKAR2A Control Vector (LP)**</td>
<td></td>
</tr>
<tr>
<td>35 - SmBiT-PRKACA Control Vector (SP)**</td>
<td></td>
</tr>
<tr>
<td>36 - NanoBiT Negative Control vector - HaloTag-SmBiT (HT)**</td>
<td></td>
</tr>
<tr>
<td>37 - pBiT1.1-N[TK/LgBiT] Vector</td>
<td></td>
</tr>
<tr>
<td>38 - pBiT1.1-C[TK/LgBiT] Vector</td>
<td></td>
</tr>
<tr>
<td>39 - pBiT2.1-N[TK/SmBiT] Vector</td>
<td></td>
</tr>
<tr>
<td>40 - pBiT2.1-C[TK/SmBiT] Vector</td>
<td></td>
</tr>
</tbody>
</table>
8 Recombinant Protein Expression and Purification

8.1 GST-βarr2 and GST expression and purification

Constructs of interest (constructs 27 and 28) were transformed in BL21-CodonPlus (DE3)-RIL E.coli bacteria and plated on LB carbenicillin plates at 37°C overnight. A single colony was inoculated into 50 mL reconstituted LB Broth Miller (BioShop LBL407.5) containing 100 µg/mL carbenicillin and grown overnight at 37°C with agitation at ≈200rpm. Two or four 4L flasks, containing each 1L of LB and carbenicillin (100 µg/mL) (for a total volume of 2 or 4 L), were inoculated with 5mL of overnight culture/flask. Following incubation at 37°C with agitation at ≈200rpm, for ≈4-5h or until O.D.600 reached ≈0.8, expression was induced by adding IPTG at a final concentration of 400 µM and the culture was again incubated for ≈4h at 37°C or overnight at 16°C.

The cells were harvested by centrifugation at 4°C (4500 x g, 20 min), and washed with 25 mL of PBS 1X/L of bacteria culture, then centrifuged again (4500 x g, 20 min) before discarding the supernatant and freezing the pellet at -20°C. Alternatively, the cells were resuspended directly in the lysis buffer (50 mM Tris pH 8.0, 300 mM NaCl, 3mM β-mercaptoethanol, supplemented with protease inhibitors (Sigma, s8830)). Cells were resuspended in 25 mL lysis buffer/L of initial cell culture. Homogenisation was performed with Avectin Emulsiflex C3 (ATA Scientific) at ≈15 000 psi at 4°C. The lysate was then centrifuged in an ultracentrifuge at 33 500 rpm for 1h at 4°C. The supernatant was then used to proceed with purification.

The cell lysate was applied at 4°C to a Glutathione Sepharose 4B (GE Healthcare, 52-2303-00 AK) column (8 mL of slurry for 4 mL of resin) equilibrated with purification buffer (50 mM Tris pH 8.0, 300 mM NaCl, 3mM β-mercaptoethanol). After a second passage the column was washed with purification buffer. Proteins were eluted using elution buffer (50 mM Tris pH 8.0, 300 mM NaCl, 3mM β-mercaptoethanol, 15mM glutathione reduced). Samples were then used for SDS-PAGE analysis to verify the expression and purification efficiency.
8.2 Mutated Akt constructs expression test

Constructs of interest (constructs 1-8 inclusively) were transformed in BL21-CodonPlus (DE3) - RIL E.coli bacterias. A single colony was inoculated into 5 mL LB broth containing 100 µg/mL carbenicillin and grown overnight at 37°C with agitation at ≈200rpm. The overnight culture O.D._{600} was measured and the culture was then diluted accordingly to O.D._{600}=0.1 in a total volume of 5mL LB containing the same concentration of carbenicillin. Following incubation at 37°C with agitation at ≈200rpm until O.D._{600} reached ≈ 0.3, expression was induced (except in the negative control sample) by adding IPTG at a final concentration of 300 µM and the culture was incubated for an additional 5h after reducing temperature at 23°C.

The cells were harvested by centrifugation at room temperature (6000 x g, 10 min), and washed with 1mL of PBS 1X supplemented with protease inhibitors (Sigma, s8830). After centrifugation (6000 x g, 10 min), cells were resuspended in 300 µL ice cold Kinase Extraction Buffer (abcam, ab65786), then flash frozen in liquid nitrogen and stored at -80°C. For cell lysis, cells were thawed and sonicated on ice. After centrifugation at 4°C (13000 rpm, 10 min.), supernatant was transferred in new tubes and samples were prepared for western blot (WB) by addition of 5X SDS loading buffer (0.25% Bromophenol blue, 500mM DTT, 50% glycerol, 10% SDS, 250mM Tris-Cl, pH 6.8).

Samples were then analysed by WB performed with 10% Tris-glycine gradient gel (Invitrogen, XP00102BOX), followed by a semi-wet transfer on nitrocellulose membrane, which was then stained with a rabbit anti-Akt Pan antibody 1:500 (BioSource, 44-609G) and a mouse anti-GAPDH antibody 1:5000 (Santa Cruz, sc-322333) as primary antibodies (Abs). IRDye 800CW Goat anti-Rabbit (Mandel, 926-32211) and IRDye 680LT Goat anti-Mouse (Mandel, 926-68020), both at 1:10000, were used as secondary Abs. The membranes were visualized with the iBright FL1000 (Invitrogen). Commercial active Akt and inactive Akt (Millipore Ltd, 14-270 and 14-276) were used as inputs.
8.3 Akt1-PH123 and βarr2 (Arr3 untagged) expression and purification

These constructs were expressed and purified by Ernst’s lab according to previously published protocols: (Vishnivetskiy et al., 2014) for Arr3 untagged and (Thomas et al., 2002) for Akt1-PH123. Briefly, purification of Arr3 untagged involved heparin chromatography, ion exchange chromatography (Q, S) and size exclusion. Purification of Akt1-PH123 involved GST-affinity chromatography, TEV cleavage on-column and TEV removal with Ni-NTA. Samples were visualised on SDS-PAGE gels stained with PageBlue Protein Staining Solution (ThermoFisher, Cat. # 24620).

9 Pulldown Assays

9.1 Pulldown assays with recombinant βarr2

Pulldown assays were performed in collaboration with Ernst’s lab, by incubation of purified recombinant GST-βarr2 and GST proteins with commercially available Akt, in inactive or active (pAkt) form (Millipore Ltd, 14-270 and 14-276). An amount of 260 nM or 2600 of GST-βarr2 or GST was incubated with 26 nM of Akt or pAkt in a final volume of 50 ul, with 25 ul of beads, in a 1.5 mL microcentrifuge tube, overnight at 4°C on a rotation wheel. Pulldown buffer was composed of 20 mM Tris, 150 mM NaCl, 20% glycerol, 1 mM EDTA, 1 mM DTT, 10 mM β-glycerophosphate, 10 mM NaF, 0.02% Brij35, pH 7.3. Beads were centrifuged at 300 x g for 5 min at 4°C, then washed 5 times with 100 ul of washing buffer (20 mM Tris, 1 mM NaCl, 20% glycerol, 1 mM EDTA, 1 mM DTT, 10 mM β-glycerophosphate, 10 mM NaF, 0.02% Brij35, pH 7.3). Proteins were eluted with 80 ul of elution buffer (20 mM Tris, 300mM NaCl, 30 mM glutathione, 3mM β-mercaptoethanol, pH 8.0) for 30 min at 4°C and transferred before addition of 20 ul of 5X SDS loading buffer.

Samples were then analysed by WB performed with 10% Tris-glycine gradient gel (Invitrogen, XP00102BOX), followed by a semi-wet transfer on nitrocellulose membrane, which was then stained with a rabbit anti-Akt Pan antibody 1:500 (BioSource, 44-69-G) and a mouse anti-GST
antibody 1:1000 (Santa Cruz, sc-138) as primary Abs. IRDye 800CW Goat anti-Rabbit (Mandel, 926-32211) and IRDye 680LT Goat anti-Mouse (Mandel, 926-68020), both at 1:10000, were used as secondary Abs. The membranes were visualized with the iBright FL1000 (Invitrogen).

9.2 Pulldown assays with recombinant βarr2 and Akt1-PH123

These experiments were performed by Ernst’s lab. Briefly, the assays were conducted in pulldown buffer (20 mM MOPS pH 7.2, 150 mM NaCl, 1mM TCEP) with 100 µM of either GST or GST-tagged Akt1-PH123 with 100 µM of βarr2 (Arr3 untagged), in 100 µL final volume, with 25 µL of glutathione sepharose 4B beads (GE Healthcare, 52-2303-00 AK). Following incubation at 4°C for 2h, wash was performed with 200 µL of pulldown buffer. Elution was then done with 50 µL of pulldown buffer containing 30 mM glutathione. Samples were analyzed on SDS-PAGE gel stained with PageBlue Protein Staining Solution (ThermoFisher, Cat. # 24620).

10 Co-Immunoprecipitation (Co-IP)

HEK 293 cells were maintained in complete media (Plain high glucose DMEM (Wisent Inc., Cat: 319-015-CL), supplemented with 2 mM L-Glutamine (Wisent, 609-065-EL), 10% FBS (Hyclone, SH303.96.03) and 1X Pen-Strep (Gibco, Cat #: 15140122)) in a 5% CO₂ incubator at 37 °C. Cells were plated in 10 cm petri dishes at 2-3 M cells/10mL/dish and transfected 24h later with PEI at a 3:1 PEI:µg DNA ratio for a total DNA amount of 17 µg, adjusted with pcDNA or salmon sperm DNA (Invitrogen, Cat no. 15632-011). The amount of plasmid DNA transfected was adjusted to obtain a ratio of 1:1 for the constructs used.

Cells were harvested 48h post-transfection in 1X PBS, then lysed in Lysis buffer (1X Protease Inhibitor Cocktail (PIC) (Sigma-Aldrich, P8340), 20 mM Tris-HCl pH 7.5, 150 mM NaCL, 1mM EDTA, 0.5 % NP-40) by rotation for 30 min at 4°C. Supernatant was diluted in Binding buffer (1X PIC, 10 mM Tris-HCl, 150 mM NaCl, 0.5 mM EDTA). Total protein concentration was assessed by DC protein assay (Bio-Rad) and samples were diluted in Binding Buffer to attain similar
concentrations in 700 µL (except for samples containing Myr-ΔPH-Akt1, were the total protein concentration was doubled to attain similar levels of the protein of interest). Co-IP was performed using GFP-nAb agarose beads (Allele Biotechnology, ABP-nAb-GFP100) with 2h incubation by rotation at 4°C. Samples were then washed with Wash buffer (1X PIC, 10 mM Tris-HCl, 500 mM NaCl, 0.5 mM EDTA) and finally eluted with Elution buffer (200 mM glycine, pH 2.5), which was neutralized by neutralization buffer (1M Tris-Base). The previous steps were elaborated following recommendations from GFP-nAb Agarose resin manual (Allele Biotechnology and Pharmaceuticals, Inc., 2013)

Samples were then diluted with 5X SDS loading buffer, boiled ≈10 min. at ≈70°C and kept at -20°C until further use. Samples were then visualised by WB performed with 4-20% gradient Tris-glycine gels (Thermo Fisher, XP04202BOX). followed by a semi-wet transfer on nitrocellulose membrane, which was then stained with different combinations of a rabbit anti-GFP antibody 1:2000 (Abcam, ab290), a mouse anti-Rluc antibody 1:5000 (Millipore Sigma, MAB4400), a mouse anti-GAPDH antibody 1:5000 (Santa Cruz, sc-322333) and a rabbit anti-βArr2 antibody 1:1000 (Novus Biologicals, NB300-587) as primary Abs. IRDye 800CW Goat anti-Rabbit (Mandel, 926-32211) and IRDye 680LT Goat anti-Mouse (Mandel, 926-68020), both at 1:10000, were used as secondary Abs. The membranes were visualized with the iBright FL1000 (Invitrogen).

11 Suspension BRET

HEK 293 cells were maintained in complete media (see section 10 above) in a 5% CO₂ incubator at 37°C, then plated in 6-well plates at 200K cells/1 mL/dish. Cells were transfected 24h later with PEI at a 3:1 PEI:µg DNA ratio, for a total DNA amount of 2 µg, adjusted with pcDNA or salmon sperm DNA (Invitrogen, Cat no. 15632-011). 48h post-transfection cells were washed in 500 µL Tyrode’s solution and then resuspended in 600 µL Tyrode’s solution (Sigma, T2145-10X1L). Cells were redistributed in triplicates in black 96-well plates for fluorescence (Costar #3915) and in white 96-well plates for luminescence and BRET (Costar#3917), with 90 µL/well. Fluorescence was read with an excitation filter at 485 nm and an emission filter at 535 nm. Coelenterazine was
added at a final concentration of 5 µM (10 µL of a 50 µM stock). 10 min. after adding coelenterazine, luminescence was read with a 480 nm emission filter (count time 0.5s) and fluorescence was read with a 540 nm emission filter. All suspension BRET assays were performed at room temperature (≈25°C) and all measurements were made with TriStar plate reader (Berthold Tech, driver version 1.03 (1.0.3.1), S/N: 21-1151, embedded version: 1.04) with ICE software (Berthold Tech, version 1.0.6.13). All BRET assay data was collected, treated and analysed with Microsoft Excel 365 and GraphPad Prism 6. A non-linear curve fit (one site binding (hyperbola)) was applied to the data.

For tests with the TATPEP, HEK 293 cells were transfected with βarr2-sp1 along with an amount of Akt1-PH148 producing BRET80. TATPEP and TATSCB were diluted in PBS supplemented with 0.1% cell culture grade BSA. Stocks of 100X were prepared to be added onto plates, in the cell culture media, at a final concentration of 100µM. The compounds were added at various combinations of timepoints by media change: 2h post-transfection, 12h pre-assay or 2h pre-assay, allowing incubation for different time periods.

12 Split-luciferase NanoBiT Assay


12.1 Design of the constructs

To design the testing pairs for this split-luciferase NanoBiT Assay, Akt1-PH48 and βarr2-sp1 were cloned into vectors from the NanoBiT PPI MCS Starter System (Promega, N2014) (constructs 37-40, inclusively). The same tag orientations as for previously confirmed constructs for BRET and Co-IPs were used. Therefore, Akt1-PH148 was tagged in C-terminal with either LgBiT or SmBiT, with a linker of GNS-GSSGGGGSGGGSSG. βarr2-sp1 was tagged in N-terminal with either
LgBiT or SmBiT with a linker GSSGGGSGGGSSG-GAQ (see section 7). The linkers used were as recommended by the system’s technical manual.

12.2 Split-luciferase NanoBiT assay

For the testing of the protocol, HEK 293 cells were maintained in complete media (see section 10 above), in a 5% CO₂ incubator at 37 °C, then plated in a white 96-well plate (Costar #3917) previously coated with poly-D-lysine, at 30K cells/100µL/well, in triplicates for each condition. Cells were transfected after 24h with PEI, at a PEI:µg of DNA ratio of 3:1, for a total DNA amount of 330 ng/well, adjusted with pcDNA or salmon sperm DNA (Invitrogen, Cat no. 15632-011). Alternatively, cells were plated in 12-well plates at 100K cells/1mL/well, then transfected after 24h with PEI, at a PEI:µg of DNA ratio of 3:1, for a total DNA amount of 1 µg, adjusted with pcDNA or salmon sperm DNA (Invitrogen, Cat no. 15632-011). After 24h transfected cells were replated in a white 96-well plate (Costar #3917) previously coated with poly-D-lysine, at 30K cells/100µL/well, in triplicates for each condition. The amount of plasmid DNA transfected was adjusted to obtain a ratio of 1:1 for the constructs used in all alternatives.

24h later Nano-Glo Live cell Reagent (Promega, N2011) was prepared and added on the 96-well plate according to the manufacturer recommendations, and luminescence measurements were made in TriStar plate reader (Berthold Tech, driver version 1.03 (1.0.3.1), S/N: 21-1151, embedded version: 1.04) with ICE software (Berthold Tech, version 1.0.6.13), at room temperature or 37 °C, for 5 or 30 min., without filter and with an integration time of 1 or 2 s per reading.
Section 3
Results

13 In vitro studies of the Akt-βarr2 interaction

As a means to investigate the role of different Akt phosphorylation sites on its interaction with βarr2, expression and purification of recombinant proteins for use in \textit{in vitro} pulldown assays was planned.

13.1 Active pAkt seems to have a better affinity for βarr2 than inactive Akt

Preliminary data from our group indicated that active pAkt had a better affinity for βarr2 than inactive Akt (see section 5.1). In order to replicate these experiments, and as a way to confirm the methods previously used, a GST pulldown assay was performed with recombinant GST-βarr2 and GST proteins and commercially available active pAkt and inactive Akt (Figure 8).

Purification of recombinant GST resulted in a single band representative of a pure protein, as observed on SDS-PAGE. Purification of the GST-βarr2, although showing fair amounts of the protein of interest, appeared to need further optimisation. Indeed, a smear possibly indicating degradation products appeared on SDS-PAGE. However, since GST-βarr2 was still clearly present in the sample, it was still used to test the pulldown assay protocol. The results seemed to go in the same direction than the preliminary data where GST-βarr2 interacted more with pAkt than Akt. However, it seems difficult to conclude with certainty with this specific experiment since the signals seemed low, therefore making the variations in Akt levels difficult to detect and quantify clearly, even when using 10 times more βarr2. Since the incomplete purification of GST-βarr2 might contribute to this by causing interference in the pulldown assay, optimisation of the production and purification of this protein seemed appropriate in hopes of obtaining more conclusive data.
Figure 8 Active pAkt seems to have a better affinity for βarr2 than inactive Akt

(A) SDS-PAGE analysis of GST and GST-βarr2 purification. While purification of GST resulted in one clear band when observed on SDS-PAGE, the same analysis revealed what seems to be degradation products for GST-βarr2, although less contamination seemed present in a second elution fraction (B) GST-βarr2 pulldown assays with commercially available active pAkt and inactive Akt suggest that the active pAkt presents a better affinity for βarr2 than the inactive Akt. n=1.

13.2 Optimisation of expression and purification of βarr2

Optimisation of the production and purification of βarr2 was conducted by Ernst’s lab. They generated an untagged version of βarr2 (Arr3 untagged) (construct 29) and optimised the purification according to previously published procedures by (Vishnivetskiy et al., 2014). Visualisation of the final elution fractions on SDS-PAGE revealed a single band at ≈44 kDa, indicating that a pure protein was obtained.

Figure 9 βarr2 expression and purification after optimization by Ernst’s lab

SDS-PAGE analysis revealing a pure βarr2 (Arr3 untagged) obtained by Ernst’s lab after optimization of the expression and purification of this protein, according to method described by (Vishnivetskiy et al., 2014).
13.3 Expression of Akt constructs is favored by removal of the PH domain

13.3.1 Cloning of pseudo-phosphorylated mutants of Akt

In parallel, cloning of pseudo-phosphorylated mutants of Akt was conducted. These constructs were destined to be used for protein expression in E.coli followed by purification of the protein. The coding sequence from an existing mouse Akt1 construct available in our laboratory (construct 23) was cloned in the vector pGEX4T-1 in that intention (construct 1). In prevision of purification, the construct was tagged at the N-terminal with a Twin-Strep-tag (Schmidt et al., 2013) and at the C-terminal with a 6His tag.

Replacement of residues T308 or S473 by either aspartic acid (D) or glutamic acid (E) has been shown to mimic phosphorylation on these residues, therefore mimicking Akt activation (Klein et al., 2005; Alessi et al., 1996). Hence, several pseudo-phosphorylated mutants were designed: T308E, S473E and a double mutant (2XM) presenting both T308E and S473E (constructs 3, 5 and 7). Additionally, some observations in the literature were noted about the fact that removing the PH domain of Akt could improve protein expression (Klein et al., 2005). Therefore, a truncated ΔPH version of each of the mutants previously described was also created (constructs 2, 4, 6 and 8).

13.3.2 Expression test of Akt mutants constructs

Verification of expression of the pseudo-phosphorylated Akt constructs was then performed by WB of bacteria lysates. The intention was to verify if the protein expression was successful before starting the purification procedures. This led to a confirmation of the difficulty to express full-length recombinant Akt in bacteria. Although both Akt and T308 constructs were successfully expressed with or without PH domain (Figure 10A), both 2XM and S473 constructs could not be successfully expressed as full-length Akt. Interestingly, removal of the PH domain allowed expression of these same constructs (Figure 10B). Incidentally, the constructs showing expression also presented a double band. Considering, that the WB was performed using unpurified cell lysates, this double band might be due to several factors such as a splice variant or truncated products, or might be an unspecific recognition from the antibodies. Nevertheless, this test was
sufficient to indicate that further studies with recombinant pseudo-phosphorylated Akt constructs might necessitate truncation of the PH domain. However, before pursuing experiments with ΔPH-Akt, it was estimated that a closer look at this PH domain would be valuable. This would serve to ensure that a potential interaction of the PH domain with βarr2 would not be overseen by truncating this same PH domain.

![Figure 10 Expression test of Akt mutant constructs](image)

**Figure 10 Expression test of Akt mutant constructs**

Expression test of Akt mutant constructs (constructs 1 to 8 inclusively). Bacterial cell lysates soluble supernatant and insoluble pellet fractions were observed on WB to confirm protein expression before proceeding to purification. While AktX and T308E constructs were expressed with or without PH domain (A), 2XM and S473E mutants could only showed expression after truncation of the PH domain. Commercially available active pAkt and inactive Akt served as inputs to confirm WB detection (bands expected at ≈ 59kDa). Expected band sizes were ≈ 62kDa for full-length constructs and ≈ 62kDa for ΔPH constructs.
13.4 Expression and purification of Akt PH domain

To verify if the PH domain of Akt might comprise a potential interaction site for βarr2, an Akt construct truncated to comprise aa 1-123, representing Akt PH domain, was generated by Ernst’s lab (construct 30), and its expression and purification were optimised following previously published procedures by (Thomas et al., 2002). This construct was tagged in N-terminal with a GST tag, but a TEV site allowed cleavage of the tag if necessary. Analysis by SDS-PAGE revealed a single band ≈14 kDa, indicating obtention of a pure protein.

Figure 11 Akt-PH domain expression and purification by Ernst’s lab

An Akt-PH domain construct was generated, expressed and purified by Ernst’s lab according to procedures described by (Thomas et al., 2002). SDS-PAGE analysis showed a resulting pure protein at ≈14 kDa.

13.5 Recombinant Akt PH domain and βarr2 show a weak interaction in vitro

To verify if there might be an interaction site for βarr2 within Akt PH domain, GST-Akt PH domain pulldown assays were performed by Ernst’s lab. On SDS-PAGE, interestingly, the PH domain of Akt seemed to interact with βarr2 (Arr3 on Figure 12). A 3A mutant of βarr2, considered as a preactivated form of βarr2 (Celver et al., 2002; Gurevich, 1998), was also successfully interacting with Akt PH domain. However, the interaction might not be strong as the βarr2/Arr3 bands remained faint even with the βarr2 3A mutant.
Figure 12 Recombinant Akt PH domain and βarr2 show a weak interaction in vitro

GST pulldown of recombinant Akt PH domain (construct 30) with untagged Arr3 (construct 29) and Arr3 3A mutant performed by Ernst’s lab. Although the interaction might be weak, since the band corresponding to Arr3 untagged remains faint in comparison to its own input, both arrestin constructs seem to interact with Akt PH domain.

14 Co-IP studies of the Akt PH domain and βarr2 interaction

14.1 Design and cloning of βarr2 and Akt constructs

Two complementary approaches were envisioned to study a possible interaction between the PH domain of Akt and βarr2: co-IP and BRET. Several constructs were then designed that could be used for both approaches. Therefore, all constructs derived from βarr2 were tagged in N-terminal with the BRET donor Rluc8. All constructs derived from Akt were tagged in C-terminal with the BRET acceptor eGFP (further simply called GFP) (BRET experiments will be described in section 15). This GFP tag would later allow Akt-derived constructs to serve as bait proteins for the co-IP experiments.
Several βarr-derived constructs (Figure 13) were created (from an existing Rluc8-βarr2-sp1 construct (construct 31) (Donthamsetti et al., 2015). This construct was used as a starting point since it featured an Rluc8 sequence that could be used as a BRET donor. The βarr2 sequence was then sequenced and characterized as a modified bovine βarr2 transcript variant (NCBI Reference Sequence: XM_005220181.4, modifications as K53R and V263M). From there, an Rluc8-βarr2 construct (construct 9) was cloned to assess the effects of removal of the sp1 sequence on the interaction. This sp1 sequence had been previously reported to favor βarr2 retention to the plasma membrane (Donthamsetti et al., 2015). Then, in line with previous experiments from our group, an ENLI construct was created (construct 11). In this construct, the aa from a presumed interaction interface of βarr2 with Akt were replaced by their equivalent aa from βarr1. Therefore, aa 150-160 of βarr2 were modified as follow: K153E, S154N, I155L and S159I. This construct was intended as a mimic of βarr1 for this region, to evaluate if any interaction would be specific to this interface and to βarr2. This might be used to replicate preliminary data where such mutations seemed to reduce the interaction of βarr2 with pAkt (data not shown). Finally, a truncated construct representing only the N-domain of βarr2 was created (construct 13), with a truncation site after residue 175. Since preliminary data identified regions of interest for Akt interaction in the N-domain of βarr2, it was hypothesized that interaction could still occur upon removal of the C-domain of the protein.

Figure 13 Schematics of βarr2-derived constructs for co-IP and BRET studies of Akt PH domain and βarr2 interaction

All βarr2-derived constructs destined for co-IP and BRET were tagged at the N-terminal with Rluc8. The different constructs were generated from an original βarr2-sp1 construct available in our lab (#31) and previously described in (Donthamsetti et al., 2015). This construct contained a modified bovine βarr2 transcript variant. Three new constructs were obtained by removal of the sp1 sequence (#9), truncation of the C-domain to keep only the N-domain (#13) and mutation of the putative interface B, believed to be interacting with Akt, to its equivalent aa of βarr1 (#11). Numbers in parentheses refer to the construct numbers as listed in Table 2 and Table 3.
For Akt constructs (Figure 14 Schematics of some of the Akt constructs for co-IP and BRET studies of Akt PH domain and βarr2 interaction, a first construct was obtained via Addgene: an Akt1-PH148-GFP (construct 32), corresponding to aa 1-148 of human Akt1, was chosen for its C-terminal GFP, which made it readily usable for BRET. Since the PH domain of Akt1 has also been limited down to aa 1-111 (Mahadevan et al., 2008), a construct truncated after residue 115 was generated in an attempt to limit regions outside of the PH domain that could overlap with the linker region between the PH domain and the kinase domain. This Akt1-PH115 construct (construct 10) was created to ensure that any interaction that might be observed would really be happening in the PH domain and not in the following linker region. Additionally, a loop on Akt PH domain appeared promising as a putative interaction site in reason of its apparent flexibility and its charges. In order to investigate if this loop might contribute to the interaction, aa 43-49 were mutated for GA repeated residues (construct 12). Then, a Myr-ΔPH-Akt1 (construct 24) corresponding to aa 131-480 of human Akt1 was also obtained from Addgene. Interestingly, the myristoylation (Myr) sequence here can serve to recruit Akt to the membrane (Udenwobele et al., 2017; McIlhinney, 1998), in the absence of the PH domain which would normally play a role in this recruitment. This Myr-ΔPH-Akt1 construct was used to generate a Myr-ΔPH-Akt1-GFP (construct 14), that could be used to verify if Akt could interact with βarr2 without its PH domain. Finally, full-length human Akt2 and Akt3 were also obtained (constructs 25 and 26). In order to compare the PH domains of the three Akt isoforms, these constructs served to generate Akt2-PH117-GFP and Akt3-PH114-GFP (constructs 15 and 16), which were designed to resemble Akt1-PH115.

Of note, a few of these constructs were tested that might require more optimisation or additional replications in the future. Therefore, some results concerning the ENLI, 43-49GA and βarr2-175 constructs (construct 11, 12 and 13, respectively) will not be included in the scope of this thesis.
Figure 14 Schematics of some of the Akt constructs for co-IP and BRET studies of Akt PH domain and βarr2 interaction

All Akt-derived constructs destined to be used for co-IP and BRET studies were tagged at their C-terminal with eGFP. At the exception of the Akt2-PH117 and Akt3-PH114 constructs, all these Akt-derived constructs were based on human Akt1, which counts 480 aa. Two constructs were obtained to represent Akt PH domain (#32) and ΔPH-Akt (#14). The PH domain of Akt was further truncated to remove the linker region sequence (#10). A loop on the PH domain was mutated in construct #16 to verify its potential role in the interaction. Numbers in parentheses refer to the construct numbers as listed in Table 2 and Table 3.
14.2 Akt1-PH48 and Myr-ΔPH-Akt1 interact with βarr2-sp1

Akt1-PH48 (construct 32), Myr-ΔPH-Akt1 (construct 14) or GFP (construct 33) were co-expressed in HEK 293 cells with βarr2-sp1 (construct 31), and cell lysates were used for a GFP co-IP. Interestingly, Akt1-PH48 showed interaction with βarr2-sp1. Of note, Myr-ΔPH-Akt1 necessitated twice as much total protein levels from the cell lysates to attain similar levels of the GFP-tagged protein of interest. This might be due to low expression levels or increased degradation of the protein. Nevertheless, it seems that, at similar levels of either Akt1-PH48 or Myr-ΔPH-Akt1, both proteins interact with βarr2-sp1.

![Figure 15 Akt-PH148 and Myr-ΔPH-Akt1 interact with βarr2-sp1 in co-IPs](image)

**Figure 15 Akt-PH148 and Myr-ΔPH-Akt1 interact with βarr2-sp1 in co-IPs**

GFP co-IP of Akt1-PH148 and Myr-ΔPH-Akt1 shows that both constructs seem to interact with βarr2-sp1. βarr2-sp1 was detected with a mouse anti-Rluc primary Ab. GFP and GFP-tagged Akt constructs were detected with a rabbit anti-GFP primary Ab. This WB is representative of n=3.
14.3 Confirmation of the PH domain interaction with βarr2

For further studies, a focus on the PH domain of Akt was applied, to try to confirm its interaction with βarr2. Several preliminary tests were conducted, with a n=1 each in view of future replications. As previously mentioned, Akt1-PH148 extends slightly further than the PH domain itself and includes part of a region linking the PH domain to the kinase domain of Akt. To assess if this region might explain the interaction previously observed, the Akt1-PH115 construct (construct 10) was used. Interestingly, this construct still showed interaction with βarr2, although less protein could be detected on WB (Figure 16A). Similarly, use of a βarr2 lacking the sp1 sequence (construct 9) still resulted in interaction, even somewhat weakened, indicating that the sp1 region was not necessary for the interaction (Figure 16B). Additionally, use of the Akt1-PH148-43-49GA construct (construct 12) failed to disrupt the interaction (Figure 16C). Since each of these tests represent n=1, further replications will be needed to verify these observations.

**Figure 16 Preliminary co-IP verifications of the Akt-PH domain interaction with βarr2**

(A) Akt1-PH115 shows a weak interaction with βarr2-sp1. (B) Both Akt1-PH148 and Akt1-PH115 interact with βarr2 even after removal of the sp1 sequence. (C) Akt1-PH148-43-49GA still interacts with βarr2-sp1. Rluc8 tagged βarr2 or βarr2-sp1 were detected with an anti-Rluc primary Ab. GFP and GFP-tagged Akt constructs were detected with an anti-GFP primary Ab. A and C were issued from the same WB, but the picture was cropped for presentation purposes.
15 BRET studies of the Akt PH domain and βarr2 interaction

The same constructs which were designed and used for co-IPs were used in BRET assays to further study the interaction between the PH domain of Akt and βarr2. Suspension BRET, where the cells are in suspension during the assay, was used to perform titration assays in HEK 293 cells co-transfected with a fixed amount of the construct serving as BRET donor and increasing amounts of the BRET acceptor construct. All these BRET assays were performed in collaboration with Ghazal Fakhfouri, previous PhD student in our group, except for section 15.3. As a reminder, all βarr2-derived constructs were tagged at the N-terminal with Rluc8 as BRET donor. Meanwhile, all Akt-derived constructs were tagged at the C-terminal with eGFP as BRET acceptor.

15.1 Akt1-PH48, but not Myr-ΔPH-Akt1, interacts with βarr2-sp1

A first BRET assay involved GFP-tagged Akt1-PH48 and Myr-ΔPH-Akt1 (constructs 32 and 24, respectively) as BRET acceptors, with βarr2-sp1 (construct 31) as BRET donor (Figure 17). Akt1-PH148 titration curve tends towards saturation as the ratio of fluorescence/luminescence increases, indicating a specific interaction with βarr2-sp1 (non linear fit, $R^2=0.80$). The titration curve for Myr-ΔPH-Akt1 does not seem to present a significant non-linear fit ($R^2=0.60$), suggesting that this construct does not seem to present saturation and would not have a specific interaction with βarr2-sp1. These results confirm an interaction between Akt1-PH148 and βarr2-sp1, as previously observed in co-IP. However, the results for Myr-ΔPH-Akt1 are in contradiction with the co-IP data indicating that this construct could interact with βarr2-sp1.

15.2 Confirmation of the PH domain interaction with βarr2

As with Co-IPs experiments, Akt1-PH115 (construct 10) was used to confirm specificity of the interaction for the PH domain (Figure 18A) while βarr2 lacking the sp1 sequence (construct 9) served to verify the effect of the removal of sp1 on the interaction (Figure 18B). Both conditions presented BRET titration curves tending towards saturation ($R^2=0.83$ for Akt1-PH115 and $R^2=0.76$ for βarr2 without sp1), indicative of a specific interaction. These results are similar to those obtained for BRET assays between Akt1-PH148 and βarr2-sp1, indicating that the interaction is not affected by further truncation of the original Akt PH domain construct or removal
of the sp1 sequence. This echoes co-IPs results in suggesting that the interaction is specific to Akt-PH domain and seemingly not influenced by addition of the sp1 sequence on βarr2.

\[
\begin{align*}
\text{βarr2-sp1 : Akt1-PH148 (n=7)} \\
\text{βarr2-sp1 : Myr-ΔPH-Akt1 (n=5)}
\end{align*}
\]

**Figure 17** Akt1-PH48, but not Myr-ΔPH-Akt1, interacts with βarr2-sp1 in BRET assays

BRET titration assay showing specific interaction of Akt1-PH148 (in black), but not Myr-ΔPH-Akt1 (in green), with βarr2-sp1.

**Figure 18** BRET confirmation of the PH domain interaction with βarr2

BRET titration assay showing specific interaction between Akt1-PH115 and βarr2-sp1 (in red) (A) and specific interaction between βarr2 lacking sp1 and Akt1-PH148 (in yellow) (B).
15.3 The PH domain of another Akt isoform, Akt2, also seems to interact with βarr2

The interaction between the PH domain of Akt and βarr2 was further investigated by comparing the PH domains of the three Akt isoforms (Figure 19). The Akt1-PH115 construct (construct 10) was therefore compared to the corresponding PH domains of Akt2 and Akt3 (constructs 15 and 16). Akt2-PH117 presented a BRET titration curve resembling Akt1-PH115, suggesting a specific interaction between Akt2-PH117 and βarr2-sp1 ($R^2=0.89$). The Akt3-PH114, although surprisingly presenting an acceptable curve fit ($R^2=0.75$), presents a lack of increase in fluorescence to luminescence ratios, even when transfected with the same DNA ratios than other constructs. This unfortunately makes this data inconclusive until further optimisation of the transfection ratios to obtain fluorescence/luminescence values allowing comparison to other constructs.

**Figure 19** BRET Akt isoforms comparison suggests that Akt2 PH domain also interacts with βarr2

BRET titration assay showing specific interaction of Akt1-PH115 and Akt2-PH117 with βarr2-sp1 (A). Assays were also performed with Akt3-PH114 and βarr2-sp1 (B). However, these experiments will need further optimisation to improve the fluorescence/luminescence ratio.
15.4 TATPEP can disrupt the interaction between Akt-PH domain and βarr2

Further experiments involved the use of TATPEP. As previously mentioned in section 5.2, a putative interaction interface was identified on βarr2 and TATPEP was modeled on the aa corresponding to this possible interface. The TAT sequence was added to render the peptides cell permeable (Bolhassani, Jafarzade and Mardani, 2017; Kim, Moodley and Liu, 2015; Zhang and Wang, 2012; van den Berg and Dowdy, 2011). BRET assays between Akt1-PH148 and βarr2-sp1 were performed, and either TATPEP or TATSCB was added to the cell culture media at different timepoints during media change: 2h post-transfection, 12h before the BRET assay or 2h before the BRET assay. TATPEP, as compared to TATSCB, showed a drastic reduction in BRET signal, suggesting a disruption of the interaction between Akt1-PH148 and βarr2-sp1. Interestingly, this effect was more pronounced, reaching around 50% reduction, when the TATPEP was added 2h post transfection (G1 and G2). Adding the TATPEP 12h pre-assay still affected the interaction, but to a lesser extent (G3 and G4). When adding TATPEP only 2 hours before proceeding for the BRET assay, the interaction was less affected, although still significantly lower than its TATSCB control.

![Figure 20 TATPEP disrupts the interaction between Akt1-PH148 and βarr2-sp1 in BRET assays](image)

Normalized percentages of BRET change with TATPEP compared with TATSCB. Data was obtained with 5 different groups (G1-G5) treated at different timepoints by changing the media and adding the compounds of interest or not in the new media. The interaction between Akt1-PH148 and βarr2-sp1 was most disrupted with longer incubation periods of TATPEP (*p < 0.05; **p < 0.01; *** p < 0.001; ****p < 0.0001)
16 Split-luciferase NanoBiT assay

16.1 General considerations and design of constructs

Previous experiments demonstrated that an interaction was occurring between the PH domain of Akt and βarr2. Furthermore, it was showed that this interaction could be successfully disrupted by addition of TATP. These results open the possibility that this interaction interface could be targeted for drug development. Given the role of an Akt-βarr2 interaction in D2R signaling mediated effects (see section 2.2.2), disruption of this interaction could lead to potential clinical benefits. However, much work is still needed in order to reach that point. As a start, further studies would be needed to develop and optimize assays or screening candidate compounds that could interfere with the Akt-βarr2 interaction. As a first step in that direction, the design of a split-luciferase assay was initiated, as this type of assay would be more amenable to potential high-throughput screenings.

The design of this assay was based on the NanoBiT Protein: Protein Interaction System developed by Promega (N2014) (Dixon et al., 2016). This assay utilises a NanoBiT reporter derived from the luciferase NanoLuc (Nluc), a small luciferase noted to present enhanced stability and increased luminescence as compared to other luciferases (England, Ehlerding and Cai, 2016). The optimised fragments for NanoBiT assays were termed LgBiT and SmBiT. The NanoBiT PPI MCS Starter System (Promega, N2014) includes vectors allowing cloning of a protein of interest with either LgBiT or SmBiT. These tags can also be placed either at the N-terminal or C-terminal. It is therefore recommended to test different combinations of tags and orientations on each protein of interest to optimise the assay. Since previous constructs showing interaction in co-IP and BRET were tagged at the C-terminal for Akt and at the N-terminal for βarr2, these orientations were conserved for the design of new constructs. Therefore, Akt1-PH148 was tagged at the C-terminal with either LgBiT or SmBiT, with the linker GNS-GSSGGGGSGGGGSSG. βarr2-sp1 was tagged at the N-terminal with either LgBiT or SmBiT with the linker GSSGGGGSGGGGSSG-GAQ. The linkers used were as recommended by the system’s technical manual.

The starter system also included a positive control in the test pair SmBiT-PRKACA and LgBiT-PRKAR2A documented in (Dixon et al., 2016). A HaloTag-SmBiT construct was also supplied to serve as a negative control. This construct can be used in combination with a protein of interest tagged with LgBiT as a way to observe background noise that could originate from non-specific
interactions. The HaloTag (HT) protein has been documented in (Los et al., 2008) and is considered as structurally stable fusion partner that is expressed throughout the cell. The various testing pairs planned are represented in Figure 21. Each testing pair for the proteins of interest will have its own negative control with the HT construct.

![Diagram of HaloTag protein interactions]

**Figure 21 Representation of the testing pairs to be used for the NanoBiT assay**

The constructs were based on Akt1-PH148 and βarr2-sp1 and correspond to constructs 17 to 20 and 34-36, inclusively. The test pair are intended to test interaction between the proteins of interest, Akt and βarr2. The negative control pairs with the HaloTag-SmBiT construct will each serve to assess potential background from nonspecific interactions for their corresponding test pair. The positive control pair will contribute to assess the protocol efficiency.

### 16.2 Protocol confirmation and orientation test

Various tests were performed to optimise the protocol. In all tests two negative controls for the test pairs were present. First, the protein of interest tagged with LgBiT was paired with the HT construct to account for possible signal from nonspecific interactions. Second, the protein of interest tagged with LgBiT was used alone to account for LgBiT basal luminescence. A mock transfection with pcDNA served as a negative control for all conditions.

The optimal conditions for the assay involved plating cells in 12-well plates, transfecting on the second day and re-plating in 96-well plates on the third day, while the assay was performed on the
fourth day. This allowed better consistency between the replicates than plating the cells directly in 96-well plates. Additionally, testing at 37 °C with 2s integration time per reading allowed optimal luminescence counts, while reading for 30 min. gave the opportunity to monitor the interaction. These conditions allowed to obtain a significant signal with the positive control pair (LP+SP), which was successfully over 200 times higher than the signals from the corresponding negative controls (LP+HT and LP) (in black and grey in Figure 22). The test pairs for the proteins of interest were then also tested, with their respective negative controls (in green and blue gradients in Figure 22). Although a minimal signal was obtained, the results seem unfortunately inconclusive for these test pairs. The assay technical manual mentions that a minimal tenfold increase, between the test pair and the corresponding negative control with HT construct, is necessary to represent a specific interaction. The data obtained did not reach this threshold, indicating that these constructs do not present a specific interaction. Increasing ratios of either constructs were tested (data not shown) and allowed for increased signals but regrettably the negative controls also showed increased signals. Overall this minimal tenfold increase from the negative controls has yet to be attained, which means further optimisation of this assay would be needed to render it compatible to test the Akt-PH domain-βarr2 interaction.
While the protocol was confirmed with the positive control (black), the test pairs for the proteins of interest (green and blue) did not yield significant results until now. As can be seen on this figure, the ratio signal to noise did not reach a significant tenfold increase that would be representative of a successful interaction. Further optimisation would be necessary in order to see if the interaction between Akt1-PH148 and βarr2-sp1 can effectively be studied with a split-luciferase NanoBiT assay. Results are n=1 with triplicates. LP: LgBiT-PRKAR2A, SP: SmBiT-PRKACA, HT: HaloTag-SmBiT, AL: Akt1-PH148-LgBiT, SB: SmBiT-βarr2-sp1, LB: LgBiT-βarr2-sp1, AS: Akt1-PH148-SmBiT.
Section 4
Discussion

17 Discussion of results

17.1 In vitro studies

In vitro studies of Akt first allowed replicating preliminary results from our group suggesting that active pAkt would have a better affinity for βarr2 than inactive Akt. This is in line with the role of this interaction downstream of D2R, where formation of an Akt-βarr2-PP2A complex allows dephosphorylation of T308 of Akt by PP2A (Beaulieu et al., 2005; Beaulieu et al., 2008). Indeed, this would suggest that active Akt can participate in the formation of this complex but may lose affinity and fall out of the complex more easily after being dephosphorylated. However, since this pulldown assay presented a weak signal, further replications might contribute to reinforce this hypothesis. Notably, use of a recombinant βarr2 of improved purity could be relevant. The βarr2/Arr3 untagged optimised by Ernst’s group presented an excellent purification. However, being untagged prevented it to be used as a bait for pulldowns, which led to a switch to use a GST tagged Akt construct as bait protein. This construct was designed to represent the PH domain of Akt.

Indeed, parallel experiments underlined the difficulty to express some Akt constructs in full-length in E.coli. Other options for protein expression exist, such as insect cells or silkworms (Fabbro et al., 1999; Maesaki et al., 2014)) and might have been an alternative to produce full-length mutant proteins. However, these methods were deemed more costly and time-consuming. An alternative was the production of a truncated form of Akt lacking its PH domain, which had been noted in the literature to improve protein expression in E.coli (Klein et al., 2005). This was confirmed in an expression test of various Akt pseudo-phosphorylated mutants, where some of the constructs did not seem to be expressed as full-length proteins, but could be produced when removing the PH domain. Before proceeding with this truncation however, it seemed relevant to verify if the PH domain of Akt could present any interaction with βarr2.
One of the methods used to verify this was pulldown assays with recombinant Akt-PH domain, performed by Ernst’s laboratory. Expression and purification of a GST-tagged PH domain of Akt was optimised. Then, used in pulldown assay with an untagged βarr2, this construct showed a weak interaction. Further controls could include the use of a ΔPH Akt, or different truncations in order to map more precisely an interaction site between Akt and βarr2. Testing of the pseudo-phosphorylated Akt mutants could also still be considered to pursue the evaluation of the effect of different phosphorylation sites on the interaction, at least in ΔPH constructs. Careful design of new constructs and thoughtful optimisation of expression and purification of recombinant proteins would still be needed in that regard. Eventually, if access to other production means becomes possible to express full-length Akt constructs, comparison of the full-length recombinant protein with its PH domain would also be relevant. In the meantime, the current pulldown assays give an indication that the PH domain of Akt presents some interaction site for βarr2.

17.2 Co-IPs

Co-IPs were used as a means to further investigate if an interaction site for βarr2 could lie within Akt PH domain. These experiments demonstrated that the PH domain of Akt, by itself, can indeed interact with βarr2. Further replications might be needed to assess with more certainty the effects of a truncation of the PH domain to aa 115 and removal of the sp1 sequence on βarr2. As of now, preliminary data suggests that these modifications might reduce the interaction affinity but do not cause a complete disruption, at least in co-IPs. The construct Myr-ΔPH-Akt1 proved to be more challenging to express than the Akt-PH domain, yielding less total protein levels and less of the construct of interest detected on WB. Reduced expression or increased protein degradation are possible causes of these low yields. It can be noted also that this construct size (69.5 kDa) is more than for PH domain (44.7 kDa), which might have influenced its expression levels. Nevertheless, use of increased amounts of cell lysate in co-IPs allowed to obtain comparable protein levels on WB and, interestingly, Myr-ΔPH-Akt1 also seemed to be able to interact on its own with βarr2 in co-IPs. This opens the possibility of multiple interaction sites between Akt and βarr2. Since two putative interfaces were identified until now on βarr2, it does not seem surprising that Akt might also present several potential interaction interfaces. Further studies will likely be needed to identify more precisely where these interfaces are located on Akt and what role each may play. Considering
the multiple functions of both Akt and βarr2, further studies could also investigate if each potential interaction site may play a different role or if several interaction sites might just be necessary to stabilise the interaction. Other interesting future possibilities could be comparisons with full-length Akt, which could lead to testing of the Akt pseudo-phosphorylated mutants in co-IPs. As of now, the co-IPs indicate an interaction between Akt PH domain and βarr2.

17.3 BRET assays

BRET assays further reinforced the hypothesis of an interaction between the PH domain of Akt and βarr2. However, results for Myr-ΔPH-Akt1 here indicate that this construct might not interact with βarr2, which is in contradiction with results obtained from co-IPs. This could be due to various factors. For example, Myr-ΔPH-Akt1 might present a very weak or transient interaction with βarr2, making it difficult to detect in BRET assays in the current conditions. Optimisation of BRET assay conditions might be interesting to verify this hypothesis. It is also possible that the GFP tag orientation of the Myr-ΔPH-Akt1 construct, or the linker attaching this tag, might be in cause. Indeed, steric hindrance or distance limitations might prevent formation of the BRET signal. Experiments placing the GFP tag in N-terminal instead of in C-terminal, or varying the linker length, could contribute to address those issues. Effects of these modifications should also be assessed in parallel in co-IPs in order to monitor closely their effects. Finally, use of an additional negative control would be valuable. Indeed, use of proteins known for not interacting together but tagged in a similar fashion than the constructs of interest could serve to generate a negative control BRET titration curve which could then be used to compare and better assess if the Myr-ΔPH-Akt1 really does not present a specific interaction.

Akt1-PH115 and βarr2 lacking sp1 didn’t seem to have a major impact on the BRET signal, as compared to Akt1-PH148 interaction with βarr2-sp1. This indicates that the interaction is specific to the PH domain of Akt, and that βarr2 doesn’t need the sp1 sequence to interact. Since this sp1 sequence has been noted to contribute to retain βarr2 at the plasma membrane (Donthamsetti et al., 2015), questions about the subcellular localisation of the interaction can surface. Considering the involvement of βarr2 with D2Rs, leading to its recruitment at cell membrane (Beaulieu and Gainetdinov, 2011), it was hypothesized that an Akt-βarr2 interaction could occur at the plasma membrane. As a reminder, Akt itself is also recruited at the membrane by its PH domain, although
this seems to be transient and short lived (Manning and Toker, 2017). One can also notice that both Akt and βarr2, can be present either at the membrane or at other intracellular pools, which might affect their functions (Peterson and Luttrell, 2017; Manning and Toker, 2017). However, the fact that the sp1 sequence does not seem necessary for the interaction might still not indicate that the interaction does not happen at the membrane. Since BRET assays are conducted in cells, it is not impossible that other receptors or plasma membrane elements might serve here to bring βarr2 at the membrane, rendering the sp1 sequence unnecessary. Alternatively, the interaction could occur at the plasma membrane and then move to another intracellular location to exert its action. While many scenarios could be hypothesized here, further studies seem relevant to evaluate the subcellular location of the interaction.

Interestingly, an interaction with βarr2 appeared to occur with the PH domain of Akt2 as well. The PH domain of Akt3 might need further optimisation to increase its expression and fluorescence/luminescence ratios to really assess if it can interact with βarr2 as well or not. This could be done either by increasing the amounts of DNA used for its transfection or by modifying the construct. For example, changing the length of the construct might help improve its expression and/or its stability, or reduce its degradation. These experiments could then be verified in co-IPs as well. To pursue studies of the different Akt isoforms, full-length constructs might also be used. Eventually, comparing the interacting PH domain sequences for similarities, or possibly non-interacting PH domain sequences for discrepancies, might help pinpoint a more precise location for an interaction with βarr2. It might also be interesting in the future to investigate if PH domains of other proteins might also interact with βarr2.

Interestingly also, TATPEP seemed to disrupt the interaction between Akt1-PH148 and βarr2-sp1. Since TATPEP corresponds to a specific putative interaction interface on βarr2, its effect suggests that the PH domain of Akt might interact on this specific potential interface, corresponding to aa 150-160 of βarr2. Additionally, the fact that the interaction can effectively be disrupted suggests that it might be targetable for future drug development. Intriguingly, the effect of the TATPEP was attenuated when given later after transfection and closer to the assay time. It can be hypothesized that the interaction is effectively prevented as soon as the proteins are expressed when the TATPEP is added sooner after transfection. However, the TATPEP might not be able to disrupt the interaction as effectively once it is formed, later after transfection. Alternatively, the TATPEP might not have the time to fully exert its effect in a short period of time pre-assay. Therefore,
dynamics of the interaction and of its disruption by TATPEP would be interesting future topics of study.

Other considerations concern improvements that could be made to the methodology used to analyse BRET data. Fluorescence/luminescence ratios are commonly used for analysis and seem to be sufficient here to identify specific interactions. Nevertheless, it has been observed that normalisation of such ratios could contribute to represent more accurately the actual levels of interacting molecules involved, which could notably improve the quantitative qualities of BRET data (Salahpour and Masri, 2007). This requires cloning of a unimolecular construct composed of the BRET donor and acceptor, in this case Rluc8 and GFP, linked together. Such a construct was successfully cloned and tested briefly in order to obtain a standard curve. However, the fluorescence values after addition of coelenterazine obtained for this construct were significantly higher than the values obtained for other assays, making them difficult to use as a proper standard. Further titrations of this construct will be needed in order to obtain signals suitable for such a purpose. Normalisation of the data could then be applied to get an improved interpretation of BRET experiments.

Another important improvement to the BRET analysis could also be made by the use of BRET$_{50}$ (Szalai et al., 2014). BRET$_{50}$ would here correspond to the value of fluorescence/luminescence ratio giving half of the maximal BRET value. In this idea, a low BRET$_{50}$ would indicate interaction while a high BRET$_{50}$ would indicate weak interaction. Interestingly, these inputs from BRET$_{50}$ could remain valuable even with a low BRET$_{max}$. Indeed, since the absolute value of BRET$_{max}$ ratio also depends on the distance between donor and acceptor in a complex, it might not be as indicative by itself of the actual dynamics of the interaction. Therefore, the BRET$_{50}$ value would be a meaningful addition to the analysis of BRET data to estimate the strength of a potential interaction and, consequently, if this interaction seems plausible or not.

### 17.4 NanoBiT assay

The NanoBiT assay was implemented with the intention to transfer the existing constructs to a system more amenable for high-throughput assays in the future. Indeed, since the interaction between the PH domain of Akt and βarr2 seemed relatively consistent across pulldown assays, co-
IPs and BRET assays, further studies seemed warranted. Furthermore, the use of the TATPEP allowed a proof of concept that this interaction can successfully be disrupted. Preliminary data from our group (see section 5.4) indicated that TATPEP, while selectively disrupting the Akt-βarr2 interaction, also mimicked some behavioral effects of LiCl on amphetamine and novelty-induced locomotion in mice. It can therefore be hypothesized that identifying a compound that could successfully inhibit this interaction might lead to clinically relevant drug candidates. A first step in hope to identify such compounds is therefore the design of an assay amenable to high-throughput screening studies.

The NanoBiT assay seemed suitable for this purpose. Split-luciferase assays are often used in high-throughput screening assays in reason of their sensitivity and simplicity. They can be implemented in living cells and luminescence can be readily generated upon addition of a substrate to the growth medium. The split fragments also present reversible assembly. These characteristics allow to observe dynamics and kinetics, as opposed to split fluorescent proteins, for example. Additionally, the split-luciferase fragments tags are usually small, unlike β-galactosidase assays for instance. This reduces possibilities of steric hindrance interfering with the interaction between the target proteins (Dixon et al., 2016; Wehr and Rossner, 2016). A drawback associated with split-luciferase reporters has been their relative instability in some conditions. It can also be noted that different split sites have been documented for each luciferase. The NanoBiT reporter comprises small fragments (17.6 kDa and 1.3 kDa) derived from the small luciferase NanoLuc, reported for its stability and bright luminescence (Hall et al., 2012). These fragments were optimised for stability and their intrinsic affinity and association constants were considered outside of the range for typical protein interactions, making them suitable for split-luciferase assays (Dixon et al., 2016).

Establishment of the protocol in our laboratory allowed to troubleshoot diverse parameters. When optimal parameters for detection of interaction of the positive control test pair (LP+SP) were attained, further testing of two putative test pairs (AL+SB and LB+AS) was added. These test pairs presented different combinations of tags on either Akt1-PH148 or βarr2-sp1. It was intended to verify which pair would yield the better signal to noise ratio, in order to select this pair for further studies. However, the results obtained did not present even any significant signal for both testing pairs. Considering that the positive control pair presented an excellent signal, the protocol itself does not seem to be the cause. Moreover, the interaction between Akt1-PH148 and βarr2 seemed consistent through three other different types of assays, making it unlikely that the lack of signal
would result from a lack of interaction. Although care was taken to follow recommendations from the technical manual associated with the NanoBiT assay, a possible solution could be to optimise the constructs used. It might be relevant, for example, to use a higher expression promoter. Indeed, the vectors issued from the NanoBiT PPI MCS Starter System (Promega, N2014) used HSV-TK as a low expression promoter. The intent stated was to avoid background noise that could result from high protein expression. However, given the results obtained, increasing expression by switching to a higher expression promoter such as CMV might be beneficial. Another possibility could be to extend the linker used for the tags. This could allow reduction of steric hindrance and might be beneficial to visualise the interaction. Considering that tests increasing protein ratios showed increased signal but corresponding increased background noise with increased DNA transfection, modification of the linker might be a preferable approach. It could also be interesting to change the tag orientations on each protein, i.e. to place tags in N-terminal of Akt and in C-terminal of βarr2. This might require removal of the sp1 sequence on βarr2. Nevertheless, it seems that careful optimisation of the assay conditions would be necessary to pursue its use for assessment of the Akt PH domain-βarr2 interaction.

18 Additional strengths and limitations

While several strengths and limitations were already discussed, some more general points can be mentioned. Overall, this work highlights a probable interaction between the PH domain of Akt and βarr2. The potential druggability of this interaction and its relevance for future clinical applications appear as advantages of these studies. Another strength resides in the confirmation of this interaction with three different methods (pulldown assays, co-IPs and BRET assays). The fact that the constructs used for pulldown assays differed from those used in co-IPs and BRET assays can be considered as both a strength and a limitation. Indeed, constructs for pulldown assays were based on mouse Akt and rat βarr2, while constructs for co-IPs and BRET assays were based on human Akt and a modified bovine βarr2 transcript variant (see section 7). While this can be a weakness in reason of possible interspecies variability, it can also turn out as a strength in that the interaction still consistently occurred even with those variations. However, an important limitation of this study could reside in the work yet to come. As mentioned previously, several optimisations,
additional replications and controls will be needed to strengthen the understanding of the mechanisms and dynamics of this interaction.

19 Future directions

The current findings likely are only the tip of the iceberg to understand optimally the interaction between Akt and βarr2. For example, discrepancies in results for the Myr-ΔPH-Akt1 raise questions on the possibility of several putative interaction interfaces. Verification of the interaction between Akt and βarr2 with mass spectrometry is another interesting possibility for the future. Another question which will have to be monitored closely is the effect of disrupting this interaction on Akt signaling. Indeed, although preliminary data did not demonstrate an effect of TATPEP on N2A cell growth, it seems relevant to keep in mind that Akt is involved in a vast array of other mechanisms. The experiments presented here nevertheless constitute a good starting point to continue to investigate the Akt-βarr2 interaction. Considering the involvement of this interaction in the mechanism of action of several antipsychotics and mood stabilizers, among which lithium, this target seems to present a definite clinical relevance. Therefore, future studies further improving understanding of the Akt-βarr2 interaction may allow targeting of this interaction for drug development. This precise targeting may even lead to develop new compounds presenting fewer adverse side effects.

20 Conclusion

This thesis underlines that the PH domain of Akt presents an interaction interface with βarr2. This was confirmed in pulldown assays, in co-IPs and in BRET assays. An interaction interface for βarr2 also appears to occur within the PH domain of another Akt isoform, Akt2. Furthermore, the interaction seems to occur at the previously identified “interface B” on βarr2 since it is competed by TATPEP, but not TATSCB. As for Myr-ΔPH-Akt1, its contradictory results would warrant
further investigations. Nevertheless, this work helped to improve the understanding of the Akt-βarr2 interface by identifying a possibly druggable interface. Hopes are that this could serve to future studies further elucidating this interaction’s mechanisms and dynamics, and that this could eventually lead to develop clinically relevant compounds to improve treatment and quality of life of patients affected by mental illnesses such as BP or SZ.
References


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Figures 1 and 5

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Figures 6 and 7