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Allopregnanolone: a neurosteroid that potentiates TRKB dimerization through BDNF

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Abstract:

The ability of the brain to change in response to environmental input forms the basis of brain development in youth and recovery from different disorders, such as depression, in adulthood. In particular, classical and fast acting antidepressants induce brain plasticity via modulation of tropomyosin receptor kinase B (TRKB) and its ligand brain-derived neurotrophic factor (BDNF). Among the molecules that induce TRKB signalling and promote neuroplasticity, allopregnanolone (ALLO) is an endogenous neurosteroid that produces antidepressant and anxiolytic effects in clinical and preclinical studies. Synthetic formulations of ALLO have been approved for the treatment of post-partum depression, though the mechanisms behind ALLO's mood alleviating effects are unclear. We hypothesized that ALLO potentiates TRKB signalling similarly to other antidepressants. We tested the effect of ALLO on TRKB dimerization by conducting protein-fragment complementation assays (PCA) in N2a cells and found that ALLO increases dimerization. We also found that ALLO's effect on TRKB dimerization is disrupted by removing TRKB's extracellular domain, or by a tyrosine to phenylalanine mutation of the Y433 residue at the transmembrane domain of the receptor. To examine the effect of ALLO on TRKB signalling, we measured phosphorylation of TRKB and downstream signalling proteins by Western Blot analysis of TRKB-expressing HEK293T, N2a, and MG87.TRKB cells, and enzyme-linked immunosorbent assays of primary cortical neurons. We did not observe an increase in TRKB activation, but we did find that ALLO increases transcription factor phosphorylation in MG87.TRKB cells. To determine whether BDNF mRNA levels are altered by ALLO, we conducted quantitative polymerase chain reaction on ALLO treated primary cortical neurons and found that a 30-minute treatment with ALLO decreases BDNF mRNA. In summary, our data shows that *in vitro*, ALLO potentiates TRKB dimerization in a BDNF-dependent manner that is similar to other antidepressants. Positive allosteric modulation of TRKB signalling might be involved in the therapeutic effect of ALLO, though further *in vivo* studies are needed.

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List of abbreviations

ALLO – allopregnanolone

BDNF – brain-derived neurotrophic factor

BSA – bovine serum albumin

Ca^{2+}/CaM – calcium/calmodulin-dependent protein kinase

CREB – cyclic-AMP response element-binding protein

DAG – diacylglycerol

DIV – days *in vitro*

DMEM – Dulbecco's Modified Eagle Medium

DMSO – dimethyl sulfoxide

ELISA – enzyme-linked immunosorbent assay

ERK – extracellular signal-regulated kinase

GABA – gamma aminobutyric acid

GAPDH – glyceraldehyde 3-phosphate dehydrogenase

HPRT – hypoxanthine guanine phosphoribosyl transferase

HRP – horseradish peroxidase

$Ins(1,4,5)P_3$ – inositol-1,4,5-triphosphate

iPlasticity – induced critical period-like neuroplasticity

LSD – lysergic acid diethylamide

MAPK – mitogen-activated protein kinase

PBS – phosphate buffered saline

PBS-T – phosphate buffered saline with Triton X-100

PCA – protein-fragment complementation assay

PI3K – phosphatidylinositol 3-kinase

PKC – protein kinase C

PLCγ – phospholipase C gamma

PPD – post-partum depression

PtdIns(4,5)P_2 – phosphatidylinositol-4,5-bisphosphate

PVDF – polyvinylidene difluoride

qPCR – quantitative polymerase chain reaction

ROI – region of interest

SDS-PAGE – sodium dodecyl sulphate and polyacrylamide gel electrophoresis

SSRI – selective-serotonin reuptake inhibitor

TBS-T – tris-buffered saline with Tween 20

TRKB – tropomyosin receptor kinase B

TRKB_ΔEC – TRKB receptor without the extracellular domain

TRKB_Y433F – TRKB receptor with tyrosine residue 433 mutated to phenylalanine

WT – wild-type

Introduction

1.1 A better understanding of antidepressant mechanisms is needed

Depression and anxiety are the two most common mental health disorders and are often precipitated or exacerbated by acute or chronic stress (Gold & Chrousos, 2002; Kendler et al., 1999). Improving treatment outcomes for these common mental disorders is a pressing matter, as they continue to be responsible for a significant portion of the global disease burden with no evidence of reduction since 1990 (Ferrari et al., 2022). Despite a long history of medication development and evidence-based interventions, the response rate to treatment with typical antidepressant medication, such as selective-serotonin reuptake inhibitors (SSRIs), rarely exceeds 50% and clinical effects typically take several weeks to appear (Pigott et al., 2023). It is still unclear exactly how antidepressant drugs contribute to recovery from stress-related mood disorders and a better understanding of the mechanisms responsible for their clinical effects could guide treatment plans and improve therapeutic outcomes.

1.2 The network hypothesis links stress, mood disorders, and recovery

Knowledge regarding antidepressant mechanisms is largely derived from animal studies, including for the long-standing monoamine hypothesis. Born out of recognition that antidepressant drugs increase brain levels of monoamines in animal models, the monoamine hypothesis proposes that mood disorders such as depression and anxiety are caused by a deficiency in monoaminergic neuromodulators and that antidepressant drugs act by replenishing them (Schildkraut, 1965). However, one of the criticisms of this theory is that although these medications rapidly increase the synaptic levels of monoamines by inhibiting their reuptake or metabolism, their clinical effects are delayed for reasons that have been unclear (Belmaker & Agam, 2008; Malhi & Mann, 2018).

A proposed alternative theory is the network hypothesis of depression, which recognizes that mood disorders are often associated with structural abnormalities within the networks implicated in depression and suggests that changes in information processing are a key component of these conditions (Price & Drevets, 2010). In support of this theory, human studies have demonstrated that both stress and depression can

lead to neuronal atrophy and cell loss in key limbic brain regions implicated in depression, including the amygdala, prefrontal cortex, and hippocampus, and that antidepressant treatment can block or reverse these effects (Duman & Monteggia, 2006; Sheline et al., 2003; Vermetten et al., 2003). Thus, recovery from stress-related mood disorders reflects the structural and functional changes in these critical neural networks that allow them to better adapt to environmental conditions, rather than resulting from an increase or a decrease in the concentration of a single molecule (Duman & Aghajanian, 2012; Krishnan & Nestler, 2010). Since meaningful changes in neuronal networks takes time, this has been proposed as an explanation for the delay in the clinical effects of antidepressants.

1.3 Antidepressants support network recovery by increasing neuroplasticity

According to the network hypothesis, antidepressants encourage these adaptive changes by increasing neuroplasticity. Neuroplasticity mediates structural and functional changes in networks, with experience tuning the networks to represent environmental input during development and underlying our ability to learn in adulthood (Hua & Smith, 2004; Katz & Shatz, 1996). During early postnatal life there are waves of critical periods where network plasticity is highly active; in adulthood these critical periods close and network plasticity becomes much more restricted (Berardi et al., 2003; Hensch, 2004; Wiesel, 1982). A reactivation of this critical period-like plasticity (iPlasticity) appears to follow from chronic treatment with the antidepressant fluoxetine, which was first observed in the visual cortex of adult mice as an ocular dominance shift in response to monocular deprivation that is normally only possible during development (Vetencourt et al., 2008). Subsequent studies have demonstrated that antidepressants produce iPlasticity also in mood-relevant networks, such as the fear extinction and aggression control circuitries (Karpova et al., 2011; Mikics et al., 2018). Activation of iPlasticity by antidepressants can facilitate the reorganization and functional recovery of a network that was miswired during development, when paired with appropriate rehabilitation and environmental input (Castrén, 2013).

This reactivation of juvenile-like plasticity in the adult brain appears to be mediated by tropomyosin receptor kinase B (TRKB) signalling; based on observations from animal studies, it is suggested to underlie the effects of antidepressants on mood (Castrén & Antila, 2017; Karpova et al., 2011; Vetencourt et al., 2008) and behaviour (Monteggia

et al., 2004; Saarelainen et al., 2003). The endogenous ligands for TRKB are brain-derived neurotrophic factor (BDNF) and neurotrophin 4 and essentially all antidepressants, including traditional SSRIs, rapid acting antidepressants and psychedelics (Moliner et al., 2023) increase the expression and signalling of BDNF through TRKB (Autry & Monteggia, 2012; Castrén & Antila, 2017; Duman & Monteggia, 2006).

1.4 Neuroplasticity and the BDNF-TRKB signalling pathway

Activation of TRKB by BDNF is a critical mediator of activity-dependent synaptic plasticity and connectivity during development as well as in the adult brain (Park & Poo, 2013; Zagrebelsky & Korte, 2014). Synthesis and release of BDNF are regulated by neuronal activity (Thoenen, 1995), which helps to selectively stabilize active synapses at the expense of inactive ones (Je et al., 2012; Yang et al., 2009). The effect of BDNF on synapse stabilization and plasticity follows from TRKB activation, which initiates intracellular signalling cascades that modulate functional synaptic properties such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor expression and trafficking to the synapse (Caldeira et al., 2007) and increased BDNF synthesis (Isackson et al., 1991; Zafra et al., 1990).

TRKB is a single transmembrane domain protein that is activated when BDNF, a dimeric ligand, binds to the extracellular domain and induces dimerization of two TRKB monomers, resulting in autophosphorylation of tyrosine residues in the intracellular kinase domain of the receptor (Cunningham & Greene, 1998; McDonald et al., 1995). Within the autophosphorylation site, double tyrosine residues Y706/707 are required for the activation of TRKB (Saarelainen et al., 2003) while Y515 at the juxta membrane domain and Y816 at the carboxyl terminus recruit docking signalling partners (Minichiello, 2009). Phosphorylated Y515 recruits and activates SHC adaptor molecules, initiating the Ras-mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI3K)-Akt pathway, while phosphorylated Y816 recruits and activates phospholipase C gamma (PLCγ)1, initiating the PLCγ-Ca^{2+} pathway (Minichiello, 2009) (Fig. 1). Activated PLCγ1 hydrolyses phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2), generating inositol-1,4,5-triphosphate (Ins(1,4,5)P_3) and diacylglycerol (DAG) (Minichiello, 2009). DAG goes on to stimulate DAG-regulated protein kinase C (PKC) isoforms, while Ins(1,4,5)P_3

promotes release of Ca^{2+} from internal stores which activates Ca^{2+}/calmodulin-dependent protein kinases (Ca^{2+}/CaM), phosphorylating cyclic-AMP response element-binding protein (CREB) and initiating gene transcription (Minichiello, 2009) (Fig. 1). Although both Ras-MAPK and PLCγ-Ca^{2+} signalling pathways are thought to activate CREB, it appears that PLCγ-Ca^{2+} is the major pathway phosphorylating CREB and acts largely independently from the Ras-MAPK pathway (Minichiello et al., 2002). Ultimately, all three signalling pathways regulate gene transcription: the PI3K-Akt pathway promotes survival and growth of neurons, the Ras-MAPK pathway promotes neuronal differentiation and growth through activation of extracellular signal-regulated kinase (ERK) and the PLCγ-Ca^{2+} pathway promotes synaptic plasticity (Minichiello, 2009), including activation of BDNF mRNA transcription (Yasuda et al., 2007).

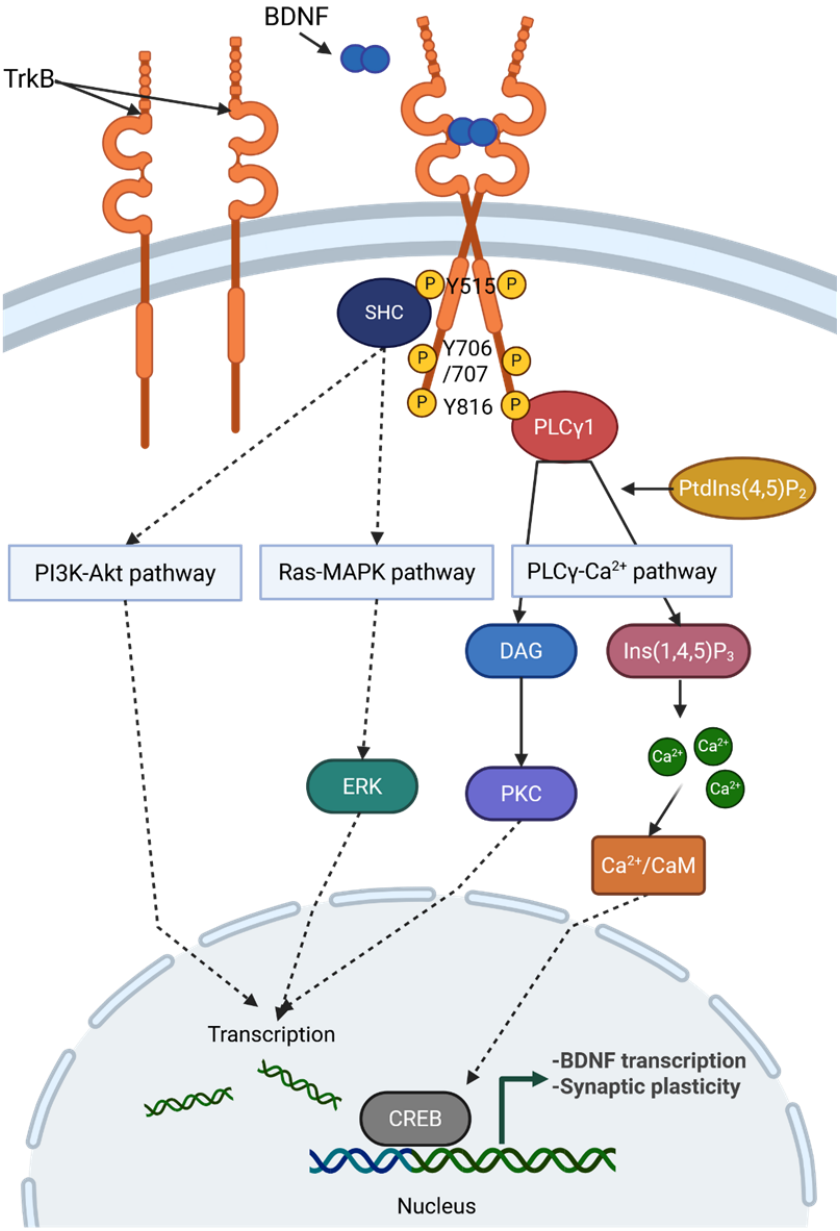

***Figure 1*. BDNF signalling pathways mediated by TRKB (simplified)**
BDNF binds to the extracellular domain of TRKB and the subsequent dimerization of TRKB monomers initiates signalling pathways (Cunningham & Greene, 1998; McDonald et al., 1995). Dimerization results in phosphorylation of several tyrosine residues, including Y706/707 situated at the autophosphorylation site (Saarelainen et al., 2003) and Y515 and Y816 which act as docking sites for signalling molecules (Minichiello, 2009). Phosphorylation of Y515 leads to the recruitment and phosphorylation of SHC adaptor molecules, initiating the PI3K-Akt and Ras-MAPK pathways, with the Ras-MAPK pathway involving ERK activation/phosphorylation (Minichiello, 2009). Phosphorylation of Y816 leads to the recruitment and phosphorylation of PLCγ1, which activates the PLCγ-Ca^{2+} pathway (Minichiello, 2009). Activated PLCγ1 hydrolyses PtdIns(4,5)P_2, generating Ins(1,4,5)P_3 and DAG (Minichiello, 2009). DAG stimulates DAG-regulated PKC isoforms, while Ins(1,4,5)P_3 promotes release of Ca^{2+} from internal stores that activate Ca^{2+}/CaM (Minichiello, 2009). Ca^{2+}/CaM go on to phosphorylate CREB, which initiates synaptic plasticity related gene transcription (Minichiello, 2009), such as BDNF mRNA production (Yasuda et al., 2007). Each signalling pathway also regulates gene transcription related to neuronal survival, growth, differentiation, and plasticity (Minichiello, 2009). Created with BioRender.com.

1.5 Antidepressants potentiate BDNF signalling by directly binding to TRKB

One of the proposed mechanisms by which antidepressants promote BDNF signalling is by directly binding to the transmembrane domain of TRKB dimers with a therapeutically relevant affinity (Casarotto et al., 2021). Direct interaction with the transmembrane domain of TRKB dimers may function as a binding site for most antidepressants as it has been observed in typical SSRIs, tricyclic antidepressants, the rapid-acting ketamine metabolite RR-HNK (Casarotto et al., 2021) and lysergic acid diethylamide (LSD) (Moliner et al., 2023). The binding of antidepressants at the transmembrane domain supports TRKB signalling by acting as a wedge, allowing it to maintain a more stable structure in the synaptic membrane and in doing so, allosterically facilitating synaptic BDNF signalling in active synapses (Casarotto et al., 2021). Facilitated BDNF signalling translates to increased phosphorylation of key tyrosine residues in the intracellular domain of TRKB. Acute antidepressant treatment increases phosphorylation of Y706/707, which are required for TRKB activation (Saarelainen et al., 2003). Acute treatment with either fluoxetine or ketamine results in enhanced phosphorylation of TRKB at Y816 residues and increased TRKB interaction with PLCγ1 (Casarotto et al., 2021); this potentiates the PLCγ-Ca^{2+} signalling pathway which is critically involved in synaptic plasticity and is reflected in the upregulation of BDNF mRNA expression that is observed following chronic fluoxetine treatment (Molteni et al., 2006; Musazzi et al., 2009). Additionally, the majority of TRKB resides in intracellular vesicles not accessible to BDNF (Du et al., 2000; Haapasalo et al., 2002; Meyer-Franke et al., 1998) and antidepressants further support BDNF signalling by promoting the relocation of TRKB to the plasma membrane (Casarotto et al., 2021). Within the transmembrane domain of TRKB, several protein residues are important in the binding of antidepressants, including Y433, V437, and S440 (Casarotto et al., 2021). Specifically, Y433 is critically involved in the binding of fluoxetine, ketamine (Casarotto et al., 2021) and LSD (Moliner et al., 2023). Antidepressant binding to the Y433 residue appears to be essential since a tyrosine to phenylalanine mutation (Y433F) disrupts the effect of antidepressants on TRKB dimerization, Y816 phosphorylation, TRKB membrane trafficking and abolishes the observed ocular dominance shift and behavioural response to antidepressants in mice (Casarotto et al., 2021).

The effect of antidepressants on TRKB signalling is also modulated by cholesterol concentrations in the plasma membrane. It was recently shown by molecular modelling that 20% cholesterol is the optimal concentration to stabilize the TRKB dimer in a signalling-competent conformation optimal for BDNF binding and receptor activation (Casarotto et al., 2021). The concentration of cholesterol modulates the orientation of the TRKB dimer in the membrane, changing the exposure of BDNF binding sites and resulting in signalling changes (Cannarozzo et al., 2021). The transmembrane domain of TRKB senses changes in cell membrane cholesterol levels due to an inverted cholesterol recognition amino acid consensus sequence (CRAC), widely conserved among different classes of proteins (Casarotto et al., 2021). Cholesterol does not compete with fluoxetine or ketamine, but increases their interaction with TRKB, which suggests that there are two distinct and cooperative recognition mechanisms for cholesterol and antidepressants within the transmembrane domain (Casarotto et al., 2021).

1.6 Allopregnanolone as an antidepressant

The behavioural effects of TRKB-binding antidepressants and structural properties of cholesterol overlap with a recently developed antidepressant, a synthetic formulation of the neurosteroid allopregnanolone (ALLO). The term "neurosteroid" refers to a class of endogenous steroids which are produced *de novo* in the central nervous system from cholesterol and modulate neuronal activity. In contrast with peripheral steroids, neurosteroids have no nuclear receptor identified and instead appear to regulate inhibitory and excitatory neurotransmission through amino acid ionotropic receptors (Rudolph et al., 2016; Schverer et al., 2018). ALLO acts as a potent positive allosteric modulator of the main inhibitory neurotransmitter, gamma aminobutyric acid (GABA), by binding within the transmembrane domain of $GABA_A$ receptors (Hosie et al., 2007). At concentrations of 10-1,000 nM, ALLO increases the frequency and duration of channel opening in response to GABA binding, resulting in increased Cl^- conduction (Carver & Reddy, 2013; Hosie et al., 2007), while greater concentrations of ALLO (1 μM and above) directly activates the gating of the chloride channels in the absence of GABA (Carver & Reddy, 2013) (Fig. 2). ALLO acts on synaptic and extra synaptic $GABA_A$ receptors (Maguire & Mody, 2008; Majewska et al., 1986; Puia et al., 1990), with the net effect being augmentation of synaptic and tonic inhibition in local brain regions respectively (Yamakura et al., 2001) (Fig. 2). Decreased levels of ALLO

have been associated with mood disorders and chronic stress (Rasmusson et al., 2019; Rasmusson et al., 2006; Romeo et al., 1998; Uzunova et al., 1998) and treatment with the antidepressant fluoxetine has been observed to normalize ALLO levels in the rodent brain as well as in the cerebrospinal fluid/serum of patients with depression (Matsumoto et al., 1999; Romeo et al., 1998; Uzunova et al., 1998). Furthermore, ALLO itself shows anxiolytic and antidepressant effects, both in humans and in preclinical models (Pinna et al., 2022).

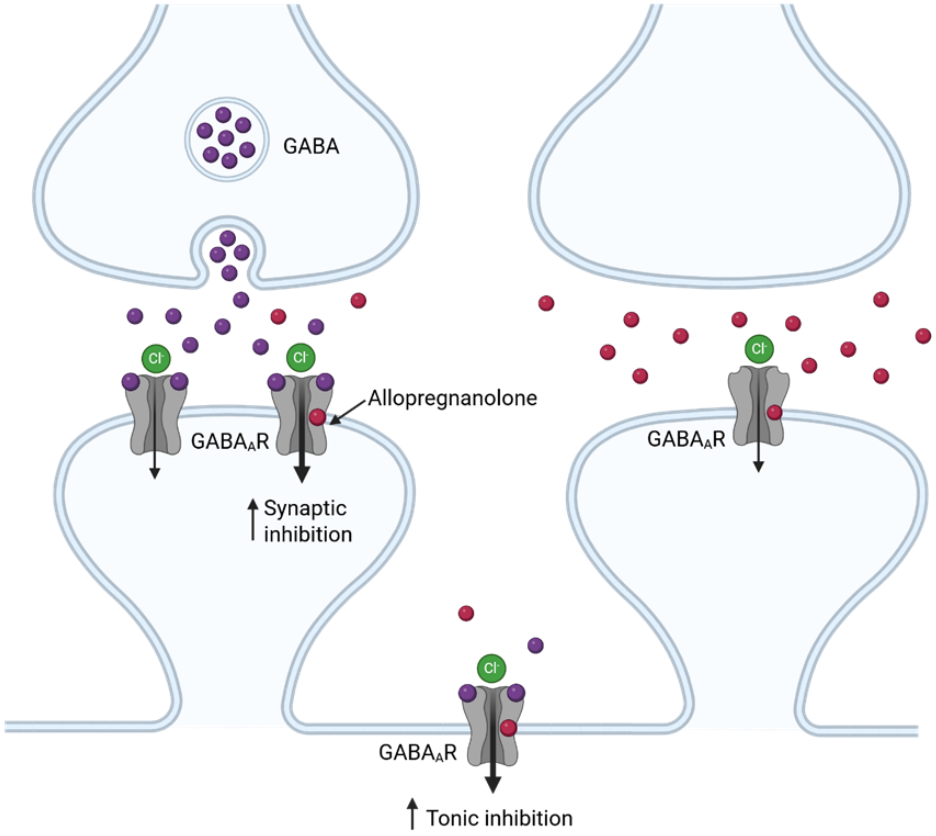

Figure 2. **Upregulation of GABAergic signalling by ALLO**
ALLO at concentrations of 10-1,000 nM allosterically enhances GABAergic signalling (Carver & Reddy, 2013). By binding within the transmembrane domain of $GABA_A$ receptors, ALLO increases the frequency and duration of channel opening in response to GABA binding, leading to increased Cl^- conductance (Hosie et al., 2007). Greater concentrations of ALLO (1 μM and above) directly activates the gating of the chloride channel without GABA (Carver & Reddy, 2013). ALLO binds to both synaptic and extra synaptic sites, with the former resulting in increased synaptic inhibition and the latter resulting in increased tonic inhibition (Yamakura et al., 2001). Created with BioRender.com.

Based on these mood alleviating effects and the observation that ALLO is rapidly depleted during post-partum (Mody, 2019), the FDA approved the synthetic ALLO formulation brexanolone for the treatment of post-partum depression (PPD) in March of 2019, under the brand name ZULRESSO (Maguire & Mennerick, 2024). PPD is a

subtype of major depressive disorder and the precipitating causes can be similar to major depression, such as chronic and acute stress exposure, frequently related to the perinatal period (Pinna et al., 2022). Brexanolone is an intravenous formulation and based on its robust antidepressant effects, an orally available compound, zuranolone, was developed (Maguire & Mennerick, 2024). Currently, the injectable ZULRESSO is no longer on the market due to commercial reasons and only the oral formulation, zuranolone, is available under the brand name ZURZUVAE. Similarly to brexanolone, zuranolone also exerts rapid and sustained antidepressant effects (Deligiannidis et al., 2023; Deligiannidis et al., 2021).

Although these drugs are intended to supplement decreased endogenous ALLO levels during post-partum, it is unclear exactly how deficits in endogenous neurosteroidogenesis contributes to mood disorders and whether binding to $GABA_A$ receptors is responsible for their therapeutic effect (Maguire & Mennerick, 2024). Considering ALLO's antidepressant properties and its structural similarities to cholesterol, facilitation of TRKB signalling is a potential mechanism mediating at least some of ALLO's antidepressant effects. A link between ALLO and BDNF has been previously identified, with antidepressant treatment upregulating both ALLO and BDNF expression in a manner that correlates with antidepressant-like effects (Nin et al., 2011). Additionally, ALLO is known to modulate BDNF levels at the hippocampus, amygdala, and hypothalamus level (Naert et al., 2007). Furthermore, in an animal study using a learned helplessness paradigm, the antidepressant-like effects of ALLO were blocked by co-infusion of TRKB antagonist ANA-12 (Shirayama et al., 2020); this suggests that ALLO might exert its antidepressant-like effects though BDNF-TRKB signalling .

Aims of the study

Considering allopregnanolone's therapeutic similarity with other antidepressants and structural similarity to cholesterol, we hypothesize that allopregnanolone allosterically facilitates BDNF signalling through similar TRKB mediated mechanisms. In this thesis I investigate how allopregnanolone affects TRKB dimerization and phosphorylation/activation of the receptor and downstream signalling proteins *in vitro,* and whether its effect is dependent on BDNF.

Methods

2.1 Experimental setup

The effect of ALLO on TRKB dimerization was studied by conducting protein-fragment complementation assays (PCA) on split-luciferase tagged TRKB-expressing N2a (mouse neuroblastoma) cells. The effect of ALLO on the phosphorylation of TRKB and downstream signalling proteins was analysed by Western Blot in TRKB-expressing N2a, HEK293T (human embryonic kidney) and MG87.TRKB (mouse embryonic fibroblast NIH-3T3 stably expressing TRKB) cells. Further analysis of TRKB phosphorylation and PLCγ1 recruitment in primary cortical neurons was conducted using sandwich enzyme-linked immunosorbent assay (ELISA). The effect of ALLO on BDNF mRNA levels was also studied in primary cortical neurons via quantitative polymerase chain reaction (qPCR).

2.2 Cell culture

2.2.1 Cell lines

Cells were obtained from ATCC and cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal calf serum (Sigma-Aldrich), 1% penicillin/streptomycin and 1% L-glutamine. I cultured the MG87.TRKB cells using the same media composition with an additional supplementation of 0.002% G418 (Institute of Biotechnology Media Kitchen, University of Helsinki). The cell lines were maintained in a cell incubator (5% CO_2, 37 °C) until reaching confluence, after which they were used for experiments or divided.

When splitting cells, I removed the old media and washed the cells with 1X phosphate-buffered saline (PBS) before detaching the cells with 2 ml of trypsin. I then collected the trypsin and cells into a falcon tube containing 8 ml of the prepared media and centrifuged them at 900 RPM for 5 minutes. Lastly, I removed the supernatant and resuspended the pellet in 10 ml of media, plating the cells at a 1:10 dilution in a 10 cm plate. When preparing the MG87.TRKB cells for experimental use, I plated the cells at a 1:9 dilution and left them to incubate for 3 days. The N2a and HEK293T cells were plated at dilutions of 1:5 to 1:7 and transfected the following day.

2.2.2 Primary cortical neurons

The primary neuronal cultures were dissected from the cortex of E18 rat embryos and plated in poly-L-lysine coated 12-well plates at a density of 2.5 x 10^5 cells/ml. Cells were maintained at 5% CO2, 37 °C in neurobasal media (Gibco) supplemented with 2% B27 (Gibco) and 1% penicillin/streptomycin by laboratory technicians as described in (Sahu et al., 2019). The cells were left undisturbed and then used for experimental procedures after a number of days *in vitro* (DIV) as specified below.

2.2.3 Transfection

I conducted cell transfection using Lipofectamine 2000 (Invitrogen), according to manufacturer's instructions. Briefly, plasmid DNA and lipofectamine were incubated separately in Opti-MEM (Gibco) for 5 minutes and then combined and incubated for an additional 30 minutes. The solution was then pipetted dropwise into the wells and the cells were left to incubate undisturbed for 48 hours (5% CO_2, 37 °C). Amount and type of DNA used for transfection and reagent volumes are experiment-specific and are specified below.

2.3 Drug treatments

For all experiments, ALLO (Tocris) was solubilized in dimethyl sulfoxide (DMSO, Sigma Aldrich) to a concentration of 5 mM and then further diluted in DMEM to achieve desired concentrations during experimental procedures. The intermediate stock of 5 mM was stored in -20 °C maximally for a month. Ketamine (Pfizer) was stored in -20 °C and diluted from a stock concentration of 40 mM to a final concentration of 10 μM using DMEM as needed. BDNF (PeproTech) was aliquoted by lab technicians, stored in -80 °C, and not refrozen after the first use.

2.4 PCA

2.4.1 Transfection

PCA was conducted to examine the effect of ALLO on TRKB dimerization in wild-type (WT) TRKB receptors, TRKB receptors lacking the extracellular domain by deletion of amino acids from position 32-429 (TRKB_ΔEC), and TRKB receptors in which the Y433 residue has been replaced with phenylalanine (TRKB_Y433F). Complementary portions of a split humanized *Gaussia princeps* luciferase (were a kind gift from Prof. Michnick (Remy & Michnick, 2006)) were cloned on the C-terminus of WT TRKB

separated by a GS linker in a pcDNA3 vector backbone (Casarotto et al., 2021), obtaining the PCA plasmids phGLuc(1C)-TRKB and phGLuc(2C)-TRKB (Fig. 3). These plasmids were further modified to produce the phGLuc-TRKB_ΔEC couple, which lack the extracellular domain, and the phGLuc-TRKB_Y433F couple in which the Y433 residue has been replaced with phenylalanine. Dimerization of TRKB brings the split luciferase in close proximity, allowing it to refold into its original and functional conformation and produce light in the presence of its substrate coelenterazine (Fig. 4). The measured bioluminescence is directly proportional to the amount of TRKB protein interacting at a given moment. I transfected N2a cells with plasmid pairs 24 hours after they had been seeded on 96-well view plates (PerkinElmer, View Plate 96) at a cell density of 10,000 cells per well A total of 100 ng of DNA (50 ng of each plasmid) was added to each well, delivered in a solution containing 0.3 µl of Lipofectamine and 20 µl of OptiMEM. Within each plate a total of 48 wells were transfected, omitting the outer edges and middle 12 wells to decrease variability and establish controls respectively.

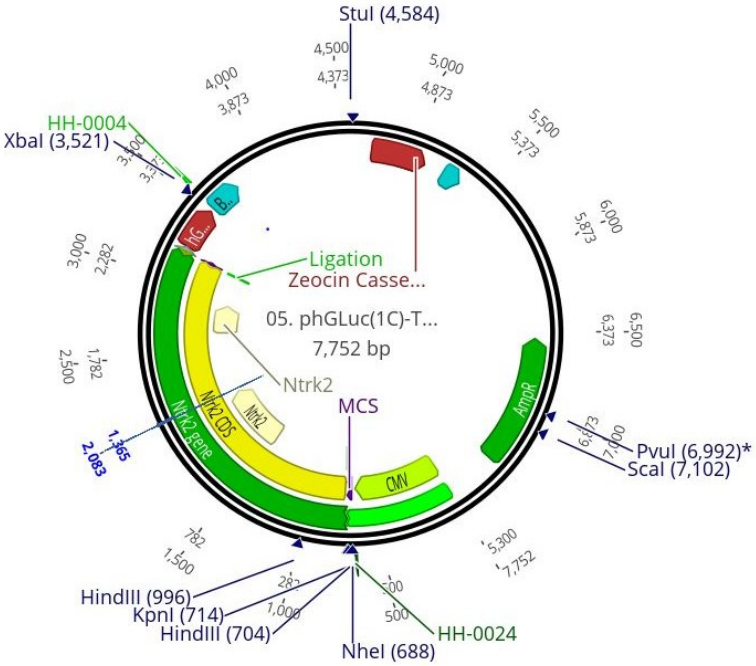

Figure 3. **Luciferase-tagged wild-type TRKB plasmid**
The plasmid vector for WT TRKB used in PCA experiments. A complementary portion of a split humanized *Gaussia princeps* luciferase is cloned onto the C-terminus of WT TRKB separated by a GS linker in a pcDNA3 vector backbone. Created with Geneious.

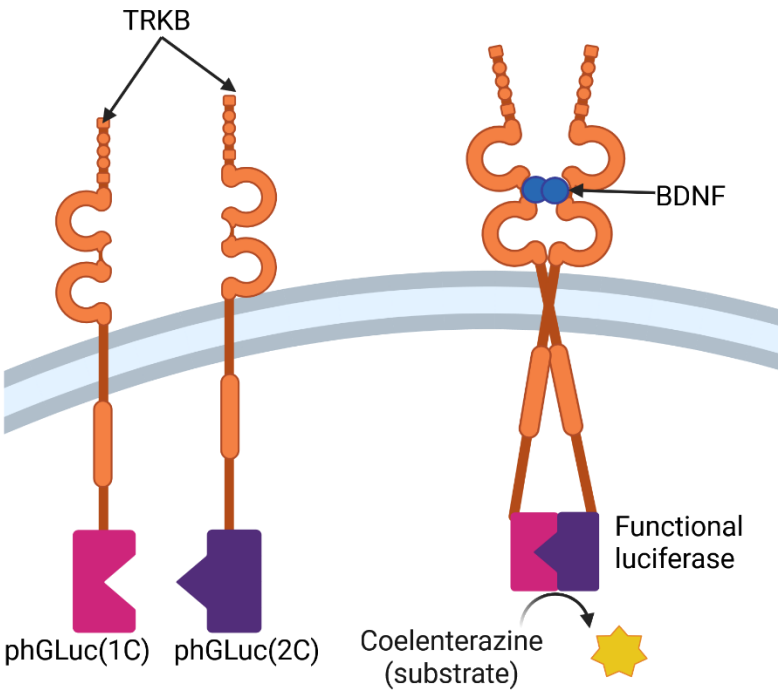

***Figure 4*. Capturing TRKB dimerization using PCA**
TRKB dimerization was captured with PCA using complementary portions of a split humanized *Gaussia princeps* luciferase (phGLuc(1C), phGLuc(2c)) cloned onto the c-terminus of a WT TRKB receptor. Dimerization of the receptors brings the split luciferase into close proximity, allowing it to refold into its functional state and produce light in the presence of its substrate coelenterazine. The luminescence produced is measured and reflects the number of dimerized receptors at that time. Created with BioRender.com.

2.4.2 Treatment and analysis

Forty-eight hours post transfection, I washed the cells with PBS and applied drug treatments using phenol red-free DMEM (30 min, 37 °C). The WT TRKB expressing cells were treated with increasing concentrations of BDNF with or without ALLO (1 μM) or increasing concentrations of ALLO alone. The treatment concentrations (ng/ml) of BDNF included 0.01, 0.02, 0.5, 20, and 50. The treatment concentrations (μM) of ALLO included 0.1, 0.5, 1, or 2. The TRKB_ΔEC expressing cells were treated with BDNF (50 ng/ml) or ALLO at concentrations (μM) of 0.1, 0.5, 1, or 2. The TRKB_Y433F expressing cells were treated with ALLO at concentrations (μM) of 0.5, 1, or 2. The PCA signal was detected using the luciferase substrate, native coelenterazine (Nanolight technology), which was directly injected into each well in the plate reader (Varioskan Flash, Thermo Scientific) at a final concentration of 25 mM. The subsequent production of light was measured for 200 ms and the final value averaged over 5 repeated measurements. The final values for each treatment condition were averaged and then subtracted from the control values (not transfected and

untreated wells). The treatment values were normalised to, and represented as a percentage of, the vehicle treated values.

2.5 Western Blot

2.5.1 Sample collection

The effect of ALLO on the phosphorylation of TRKB at Y816 and Y706/707 residues, as well as CREB and ERK, were measured by Western Blot using TRKB-expressing MG87.TRKB, N2a and HEK293T cells. I transfected N2a and HEK293T cells to over-express GFP-tagged full-length TRKB (as was done in Casarotto et al. (2021)) 24 hours after they had been seeded at a cell density of 100,000 per well in 12-well plates by laboratory technicians. A total of 1 µg of DNA was added to each well, delivered in a solution containing 2 µl of Lipofectamine and 100 µl of OptiMEM. N2a and HEK293T cells were treated 48 hours post transfection and MG87.TRKB cells were treated 72 hours after I had seeded them at a cell density of 100,000 per well in 12-well plates. This ensured that all cells were treated on day 3 after being seeded, when they had reached 90-100% confluency. I treated the cells with ALLO (1 µM, 15 minutes or 2 hours) or ketamine (10 µM, 15 minutes) at 37 °C. Untreated cells were used as a negative control. Next, I placed the cells on ice, washed them with ice-cold PBS, and lysed the cells by applying ice-cold lysis buffer (0.67% 3 M Tris-HCL pH 8.0, 2.74% 5 M NaCl, 9.6% 0.5 M NaF, 1% Nonidet P-40, 10% glycerol, 2% 100 mM NA_3VO_4, 4% 25x complete protease inhibitor mix, in MilliQ-H_2O) and scraping the wells using a cell scraper. The lysates were collected and centrifuged for 7 minutes at 14000 RPM, 4 °C. The supernatant was transferred to clean tubes and stored at -20 °C.

2.5.2 Protein quantification

To obtain the protein concentration of the lysates, I used the DC Protein Assay kit (BioRad) according to manufacturer's instructions. First, I prepared standard dilutions of bovine serum albumin (BSA, Sigma-Aldrich) from 0 to 2 mg/ml in sterile water. In a transparent 96-well plate, I added the standard dilutions and lysates in triplicate (diluted 50% with sterile water to ensure their protein concentration remained within the standard curve). After adding the reagents and incubating for 15 minutes, a colorimetric analysis was obtained using the plate reader, which measured each well for 100 ms at 750 nm. From this data, the values from the standard dilutions were averaged and plotted to create a standard curve. The protein concentration of the

lysates in µg/µl were obtained by interpolating the colorimetric values within the standard curve.

2.5.3 Electrophoresis

I submitted the lysates to sodium dodecyl sulphate and polyacrylamide gel electrophoresis (SDS-PAGE) to separate the proteins according to their molecular weights. When preparing the samples for electrophoresis, the volume of lysate in each sample was determined based on the previously ascertained protein concentrations to ensure that each sample contained the same amount of protein. The total amount of protein per sample varied between 30-38 µg depending on lysate protein concentration per experiment. In addition to the lysate, each sample was prepared with 25% sample buffer (4X NuPAGE LDS, Invitrogen) and if necessary, MilliQ H_2O to attain equal sample volumes. I then added β-mercaptoethanol (1 µl/ml, Sigma-Aldrich) to the samples and heated them at 95 °C for 5 minutes. Next, I loaded the samples and a protein ladder (Precision Plus Protein Dual Colour Standards, Bio-Rad) onto a polyacrylamide gel (NuPAGE Bis-Tris 4-12%, Invitrogen) with SDS running buffer NuPAGE MOPS (1:20 in MilliQ H_2O, Invitrogen) and ran the gel at 160V for 80 minutes at room temperature. Upon completion, I transferred the proteins to a polyvinylidene difluoride (PVDF) membrane using the semi-dry transfer method. First, I activated the PVDF membrane by soaking it in methanol. Then, the membrane, gel and blotting papers were soaked in transfer buffer (0.3% Tris, 1.44% glycine, 20% methanol, in MilliQ H_2O) for 2-3 minutes before assembling the sandwich. Within the transfer tray/cassette the PVDF membrane, gel, and blotting papers were assembled into a sandwich, with air bubbles gently rolled out between each layer. The transfer was conducted at 25V for 30 minutes at room temperature (Trans-Blot Turbo Transfer System, Bio-Rad).

2.5.4 Protein visualisation

Primary antibodies were used to target proteins of interest: TRKB, phosphorylated TRKB at Y816 (TRKB.pY816) and Y706/707 (TRKB.pY706/707) residues, CREB, phosphorylated CREB (pCREB), ERK, phosphorylated ERK (pERK), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for protein normalisation. Secondary antibodies conjugated with horseradish peroxidase (HRP) and a

chemiluminescent substrate were used to visualise protein bands. A list of primary and secondary antibodies used can be found in Table 1.

To prevent unspecific antibody binding, I first blocked the membrane using 1.5 g of skimmed milk powder (Valio) in 50 ml of tris-buffered saline with Tween 20 (TBS-T, 1:1000) for 1 hour at room temperature. After blocking, I washed the membrane with TBS-T (3x5 minutes) and incubated it with a primary antibody (1:1000 in TBS-T with 0.1% NaN_3 to prevent bacterial growth) overnight at +4 °C. Primary antibodies were stored at +4 °C and recycled. The following day, I collected the antibodies, washed the membrane with TBS-T (3x5 minutes), and then incubated it with a secondary antibody (1:5500 in TBS-T, prepared fresh) for 1 hour at room temperature. The membrane was then washed with TBS-T (3x5 minutes), placed between two sheets of plastic, and Western Blotting substrate (Pierce ECL Plus, Thermo Scientific) was pipetted on top of the membrane. The excess liquid and air were rolled out and the chemiluminescence was captured via imaging (Syngene G:BOX). A total of 10 images were taken at increasing exposure times. From these 10 images, the one with the brightest yet unsaturated protein bands was chosen for analysis. I repeated this process for each primary antibody, beginning with blocking of the membrane. The phosphorylated version of each protein was imaged before total protein to avoid potential signal loss, since the phosphorylated version is less abundant, and the membrane had to be stripped between identical molecular weights. I stripped the membrane of primary antibodies by incubating it in stripping buffer (2.08% 3M Tris pH 6.8, 10% 20X SDS, 0.7% β-mercaptoethanol, in MiliQ H_2O) for 5 minutes in a 60 °C water bath. Afterwards, the membrane was washed with TBS-T (3x7 minutes). The stripping buffer was recycled maximally 3 times. When the origin host of the primary antibody differed, I used a gentler stripping method intended to remove only the secondary antibody and ideally leave the protein underneath untouched. This stripping of secondary antibodies was done by incubating the membrane in H_2O_2 at +37 °C for 15 minutes, after which the membrane was washed with TBS-T (3x5 minutes).

2.5.5 Image analysis

The images were analysed in 16-bit and colour inverted (so that the protein bands were the darkest part of the image) using ImageJ (version 1.54k). I drew a rectangular region of interest (ROI) large enough to encompass the biggest protein band and used the

same size ROI for all protein bands, avoiding any overlap. The mean grey area was then calculated for each ROI and used for analysis. I then used the values for GAPDH to obtain normalisation factors, which were used to normalise all proteins of interest (both phosphorylated versions and total protein). Using these normalised values, the proteins were expressed as a ratio of phosphorylated version/total protein. These ratios were then calculated as a percentage of the control.

2.6 ELISA

The sandwich ELISA was conducted using primary cortical neurons to further examine ALLO effects on TRKB phosphorylation. First, I coated a 96-well high-binding plate (OptiPlate, 96 HB) with primary antibodies against TRKB.pY816, TRKB, or PLCγ1 (1:500 in carbonate buffer) by incubating the wells overnight in +4 °C. I then washed the wells with 100 µl of 0.1% Triton X-100 in 1X PBS (PBS-T) and blocked the wells to prevent unspecific binding to antibodies with 100 µl of 3% BSA in PBS-T for 2 hours at room temperature. I then treated the neurons at 12 DIV in triplicate with either ALLO (2 µM or 0.5 µM) or BDNF (50 ng/ml). The cells were left to incubate for 30 minutes (37 °C), after which I placed the cells on ice and washed them with 1000 µl of ice-cold PBS. I then added 300 µl per well of lysis buffer (as used for Western Blot) and scraped the wells using cell scrapers. The lysate was collected and centrifuged for 7 minutes at 14000 RPM, 4 °C. The supernatants were then transferred to clean tubes and each sample was pipetted in triplicate (80 µl of lysate per well) onto the antibody-coated plate, covered with parafilm, and incubated overnight in +4 °C. 24 hours later, I washed the wells 3 times with 100 µl of PBS-T and then incubated the wells with secondary antibodies against TRKB, TRKB.pY816, or PLCγ1 (1:2000 in PBS-T with 3% BSA). The wells were covered with parafilm and incubated overnight in +4 °C. I then washed the wells 3 times with PBS-T and incubated them with a tertiary HRP-conjugated antibody (1:5000 in PBS-T with 3% BSA) for 2 hours at room temperature. The antibodies used can be found in Table 1. Lastly, the wells were washed 3 times with PBS-T, incubated with Western Blotting substrate (Pierce ECL, Thermo Scientific) for 10 minutes, and the subsequent chemiluminescence in each well was measured for 1000 ms using the plate reader. The triplicate values for each sample were averaged, and then these values were averaged for each treatment condition. The final values were then calculated as a percentage of the control values.

***Table 1*. Materials list for antibodies used in experimental procedures**

Complete list of antibodies used in Western Blot and ELISA experiments

Antibody	Source	Identifier
anti-TRKB	R&D Systems	Cat#AF1494
anti-PLC-gamma1	Cell Signalling Technology	Cat#5690S
anti-TRKB-pY816	Cell Signalling Technology	Cat#4168S
anti-TRKB-pY706/707	Cell Signalling Technology	Cat#4621S
anti-phospho-CREB	Cell Signalling Technology	Cat#9198S
anti-CREB	Cell Signalling Technology	Cat#4820S
anti-phospho-MAPK (Erk1/2)	Cell Signalling Technology	Cat#9101S
anti-MAPK (Erk1/2)	Cell Signalling Technology	Cat#9102S
anti-GAPDH	Abcam	Cat#AB8245
HRP-conjugated anti-Rb IgG	Bio-Rad	Cat#1705046
HRP-conjugated anti-Ms IgG	Bio-Rad	Cat#170-6516
HRP-conjugated anti-Gt IgG	Invitrogen	Cat#61-1620

2.7 qPCR

A qPCR was conducted using primary cortical neurons to assess the effect of ALLO on BDNF mRNA levels. Primers for assessing levels of BDNF mRNA following ALLO treatment were synthetized by Sigma-Aldrich. BDNF primers were: forward, CGATGCCAGTTGCTTTGTCTTC; reverse, AGTTCGGCTTTGCTCAGTGG. Hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as housekeeping gene: forward, GGGCTTACCTCACTGCTTTCC; reverse, CTAATCACGACGCTGGGACTG. Neurons were treated at 10 DIV with 2 μM ALLO for 30 minutes or with the vehicle DMSO. Following treatment, I collected the cell lysates as before (previously described in 2.5.1 sample collection), which were then stored at -20 °C. I performed RNA isolation from lysates using the PureLink RNA Mini kit (Thermo Scientific) and reverse transcription of RNA was carried out with LunaScript RT SuperMix Kit (New England Biolabs), according to manufacturer's instructions. SYBR Green Maxima kit (Thermo Scientific) and BioRad CFX Opus 96 PCR cycler were used to detect relative mRNA amounts. Analysis was performed using the 2ΔΔCT method in Microsoft Excel.

2.8 Statistical analysis

Graphpad Prism (version 10.5) was used for one-way and two-way ANOVA analysis, unpaired t-tests, and Dunnett's and Tukey's *post hoc* multiple comparisons. Detailed statistics can be found in figure legends.

Results

3.1 Allopregnanolone potentiates TRKB dimerization

To investigate the effect of ALLO on TRKB dimerization, I conducted PCA experiments using N2a cells transfected to express split-luciferase tagged TRKB. The cells were treated for 30 minutes with different concentrations of ALLO or BDNF (50 ng/ml). This high concentration of BDNF was used since lower/typical concentrations of BDNF (10 ng/ml) had not been producing the expected TRKB response in the lab. The results show that high concentrations of ALLO (2 μM) and BDNF increase TRKB dimerization (Fig. 5). To elucidate whether ALLO potentiates the effect of BDNF on TRKB dimerization, the experiment was repeated but altered so that the cells were treated with different concentrations of BDNF either with or without ALLO (1 μM). The presence of ALLO potentiated the effect of BDNF on TRKB dimerization, but only at high concentrations of BDNF (50 ng/ml) and BDNF alone otherwise failed to induce TRKB dimerization (Fig. 6). To further investigate whether the effect of ALLO on TRKB dimerization is dependent on BDNF, the experiment was repeated using split-luciferase tagged TRKB_ΔEC expressing cells, which are unable to bind BDNF, and treating them with different concentrations of ALLO or BDNF (50 ng/ml). The results show that neither ALLO nor BDNF increased TRKB dimerization, indicating that the extracellular domain of TRKB is required for ALLO's effect on TRKB dimerization (Fig. 7a). Lastly, to assess whether the TRKB residue Y433 which is important in antidepressant binding is also critical for ALLO's effect on TRKB, the experiment was repeated once more using split-luciferase tagged TRKB_Y433F expressing cells, where the Y433 residue is converted to phenylalanine. After treating the cells with increasing concentrations of ALLO, the results shows that the TRKB Y433 residue is required for ALLO's potentiating effect on TRKB dimerization (Fig. 7b).

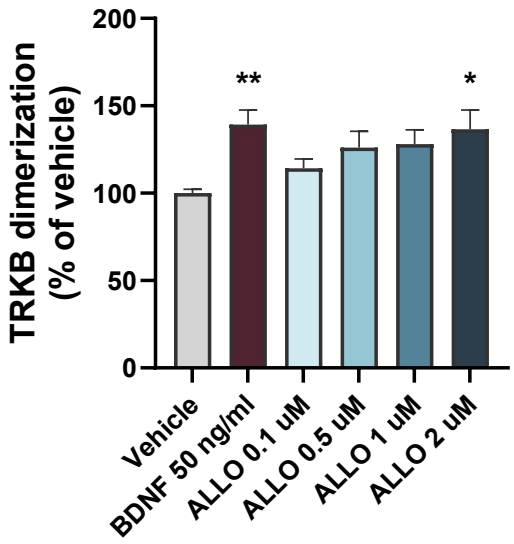

Figure 5. **ALLO treatment increases TRKB receptor dimerization**
N2a cells transfected to express split-luciferase tagged WT TRKB were treated with BDNF or increasing concentrations of ALLO for 30 minutes and submitted to PCA (n = 28/group). The presence of ALLO influences dimerization of TRKB [treatment: $F(5, 162) = 3.41$, $p = 0.005$]. ALLO concentrations of 2 μM significantly increases TRKB dimerization [$p = 0.017$, 95% C.I. = (-69.15, -4.153)]. Data expressed as mean ± SEM of percentage from control (vehicle treated) group. *p<0.05, **p<0.01 from the control group, one-way ANOVA followed by Tukey's post hoc.

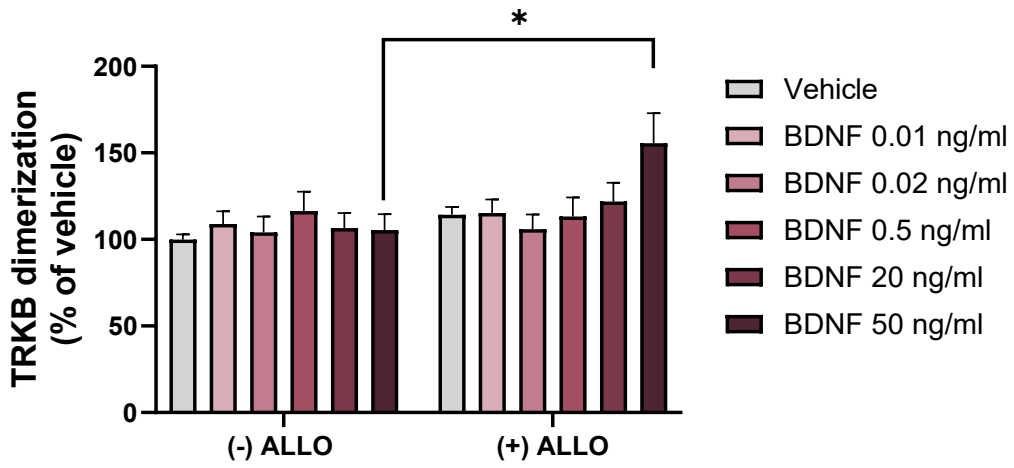

Figure 6. **ALLO potentiates BDNF induced dimerization of TRKB**
N2a cells transfected to express split-luciferase tagged WT TRKB were treated with increasing concentrations of BDNF either alone ((-) ALLO) or alongside 1 uM ALLO ((+) ALLO) and submitted to PCA (n = 28/group). The presence of ALLO influences BDNF induced dimerization of TRKB [ALLO +/-: $F(1, 304) = 6.23$, $p = 0.013$]. At BDNF concentrations of 50 ng/mL, ALLO potentiates TRKB dimerization [$p = 0.019$, 95% C.I. = (-96.11, -4.218)]. Data expressed as mean ± SEM of percentage from control (vehicle treated (-) ALLO) group. *p<0.05, two-way ANOVA followed by Tukey's post hoc.

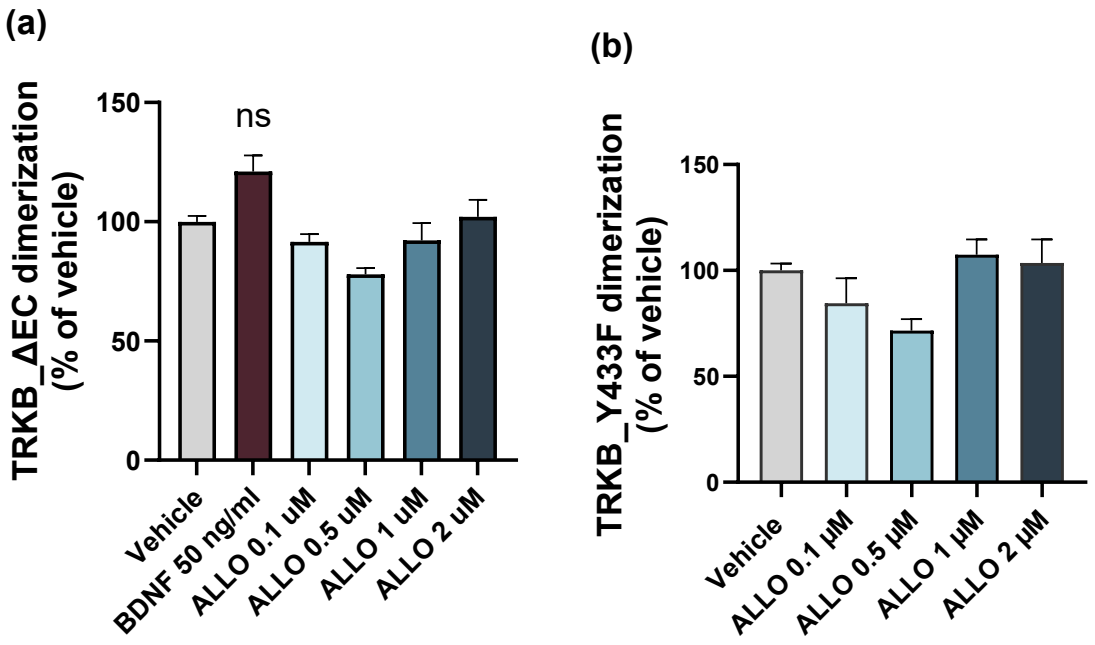

***Figure 7*. Extracellular domain and tyrosine residue 433 of TRKB are required for receptor dimerization effects of ALLO**

N2a cells transfected to express split-luciferase tagged TRKB_ΔEC or TRKB_Y433F were treated with BDNF or increasing concentrations of ALLO and submitted PCA (a) The extracellular domain of TRKB mediates the increase in TRKB dimerization in response to BDNF or ALLO [treatment: $F(5, 160) = 7.094$, $p<0.0001$] (n = 28/group). (b) The tyrosine residue at position 433 of TRKB mediates the increase in TRKB dimerization in response to ALLO [treatment: $F(5, 42) = 2.748$, $p = 0.0309$] (n = 8/group). Data expressed as mean ± SEM of percentage from control (vehicle treated) group. One-way ANOVA followed by Tukey's post hoc.

3.2 Allopregnanolone and intracellular TRKB signalling

To address the question of whether ALLO treatment increases TRKB activation or otherwise potentiates intracellular signalling, the phosphorylation of TRKB and its downstream signalling proteins ERK and CREB were quantified via Western Blot. To account for variability in TRKB expression and signalling, this was repeated in 3 cell lines; N2a and HEK293T cells were transfected to transiently express TRKB, and MG87.TRKB cells stably express TRKB. For transfection, N2a cells were chosen because they express a small amount of BDNF (Haapasalo et al., 1999) and have a neuron-like morphology, while HEK293T cells were included as they are a routinely used and readily available cell line that proliferates fast and transfects well. To account for potential differences in TRKB expression when transiently transfected, MG87.TRKB cells were included since they stably express TRKB. Although HEK293T and MG87.TRKB cells do not express BDNF, their TRKB receptors show basal

dimerization due to TRKB overexpression; this allows us to study the effect of drug treatments on TRKB signalling even in the absence of BDNF.

The cells were treated with ALLO (1 μM) for either 15 minutes or 2 hours, or with ketamine (10 μM) for 15 minutes as a positive control. Despite the increased TRKB dimerization observed at a 2 μM concentration of ALLO, a 1 μM concentration was chosen here due to the observation that ALLO is able to directly gate $GABA_A$ receptors at concentration of 1 μM and above (Carver & Reddy, 2013) and we wanted to ascertain whether ALLO could affect BDNF signalling at this lower concentration. The treatment was applied for 15 minutes or 2 hours to account for any potential direct and indirect effects on TRKB signalling respectively. Ketamine was used as a positive control due to BDNF functionality issues in the lab and a 15 minute treatment with a 10 μM concentration has been previously shown to increase TRKB phosphorylation at Y816 residues (Casarotto et al., 2021). In MG87.TRKB cells, ketamine and ALLO treatment increases phosphorylation of CREB (Fig. 8c) but not TRKB (Fig. 8a,b) or ERK (Fig. 8d). CREB phosphorylation is elevated after 15 minutes of ALLO treatment and remained elevated after 2 hours of treatment. In transfected N2a cells, ALLO does not increase phosphorylation of TRKB (Fig. 9a,b), CREB (Fig. 9c), or ERK (Fig. 9d). In transfected HEK293T cells, neither ALLO nor ketamine increases phosphorylation of TRKB (Fig. 10a,b) or CREB (Fig. 10c) and phosphorylation of ERK is increased by ketamine but not ALLO (Fig. 10d). Since we were unable to see any effect on TRKB phosphorylation using Western Blot, we investigated this matter once more using the more sensitive sandwich ELISA with different ALLO concentrations. Primary cortical neurons were treated with ALLO (0.5 or 2 μM) or BDNF (50 ng/ml) for 30 minutes and phosphorylation of TRKB at Y816 residues and recruitment of PLCγ1 was measured. Neurons were treated at DIV 12 to ensure that the synaptic machinery had developed. However, preliminary results show ALLO does not increase TRKB activation via Y816 phosphorylation or PLCγ1 recruitment (Fig. 11a,b,c). BDNF treatment successfully increased TRKB phosphorylation and PLCγ1 recruitment, but only when the capture antibody was against TRKB (Fig. 11b,c) and not against phosphorylated TRKB (Fig. 11a). Lastly, to investigate whether ALLO treatment affects BDNF production, BDNF mRNA was measured in primary cortical neurons by qPCR. Neurons were treated at DIV 10 with 2 μM ALLO, as this was the treatment

concentration that showed increased TRKB dimerization. Results show that treatment with ALLO for 30 minutes results in a decrease in BDNF mRNA (Fig. 12).

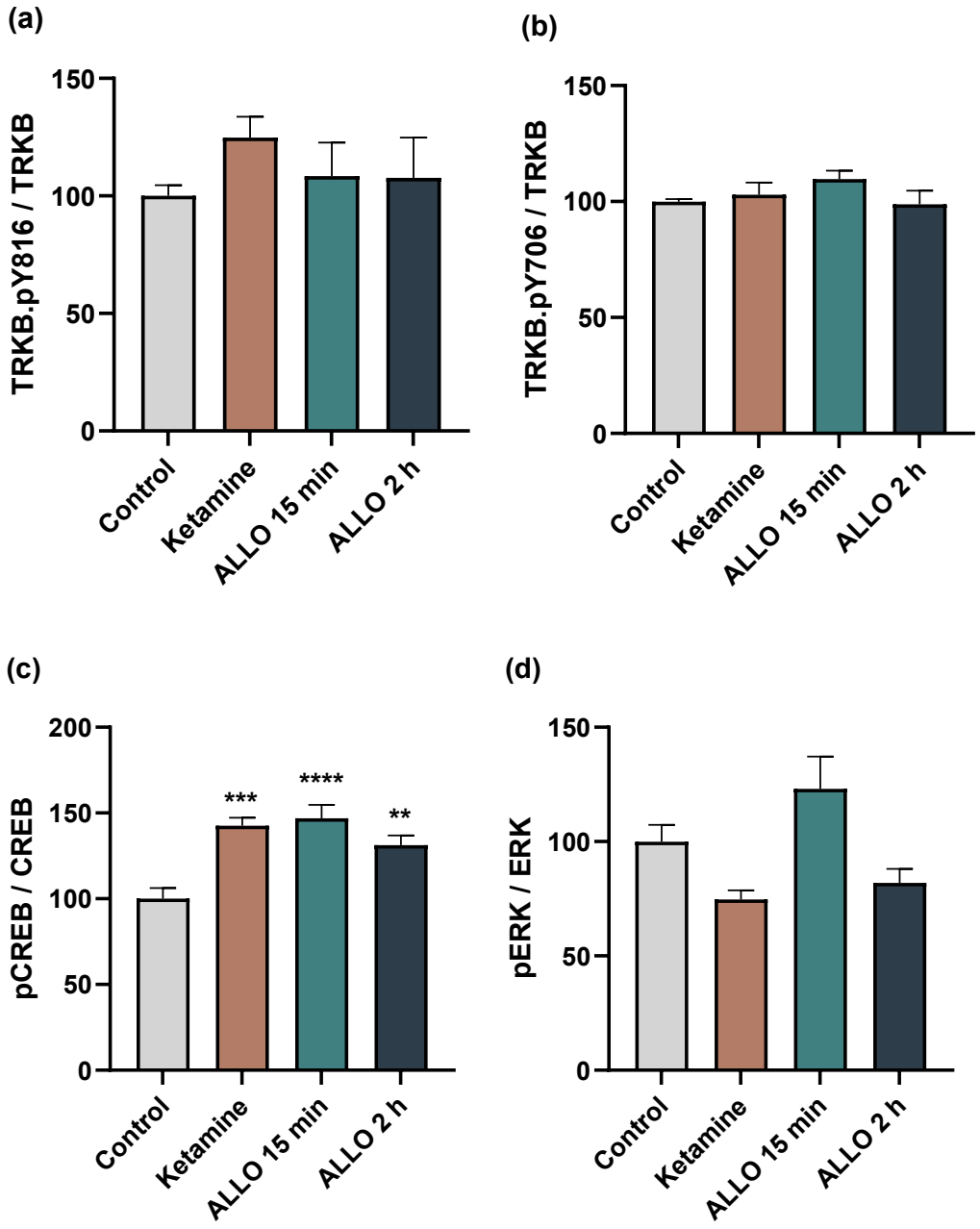

Figure 8. **ALLO increases CREB phosphorylation in MG87.TRKB cells**
MG87.TRKB cells were treated with ketamine (10 uM/15 minutes) or ALLO (1 uM/15 minutes or 1 uM/2 hours) and the amount of phosphorylated TRKB, CREB and ERK determined by Western Blot (n = 6/group). (a) Treatment with either ALLO or ketamine did not increase phosphorylation of TRKB at Y816 residues. (b) Treatment with either ALLO or ketamine did not increase phosphorylation of TRKB at Y706/707 residues. (c) Both ketamine and ALLO treatment increase phosphorylation of CREB [treatment: $F(3, 20) = 11.78$, $p = 0.0001$]. (d) There was no effect on phosphorylation of ERK with either ketamine or ALLO treatment. Data expressed as mean ± SEM of percentage from control. **p<0.01, ***p<0.001, ****p<0.0001 from the control (untreated) group, one-way ANOVA followed by Dunnett's post hoc.

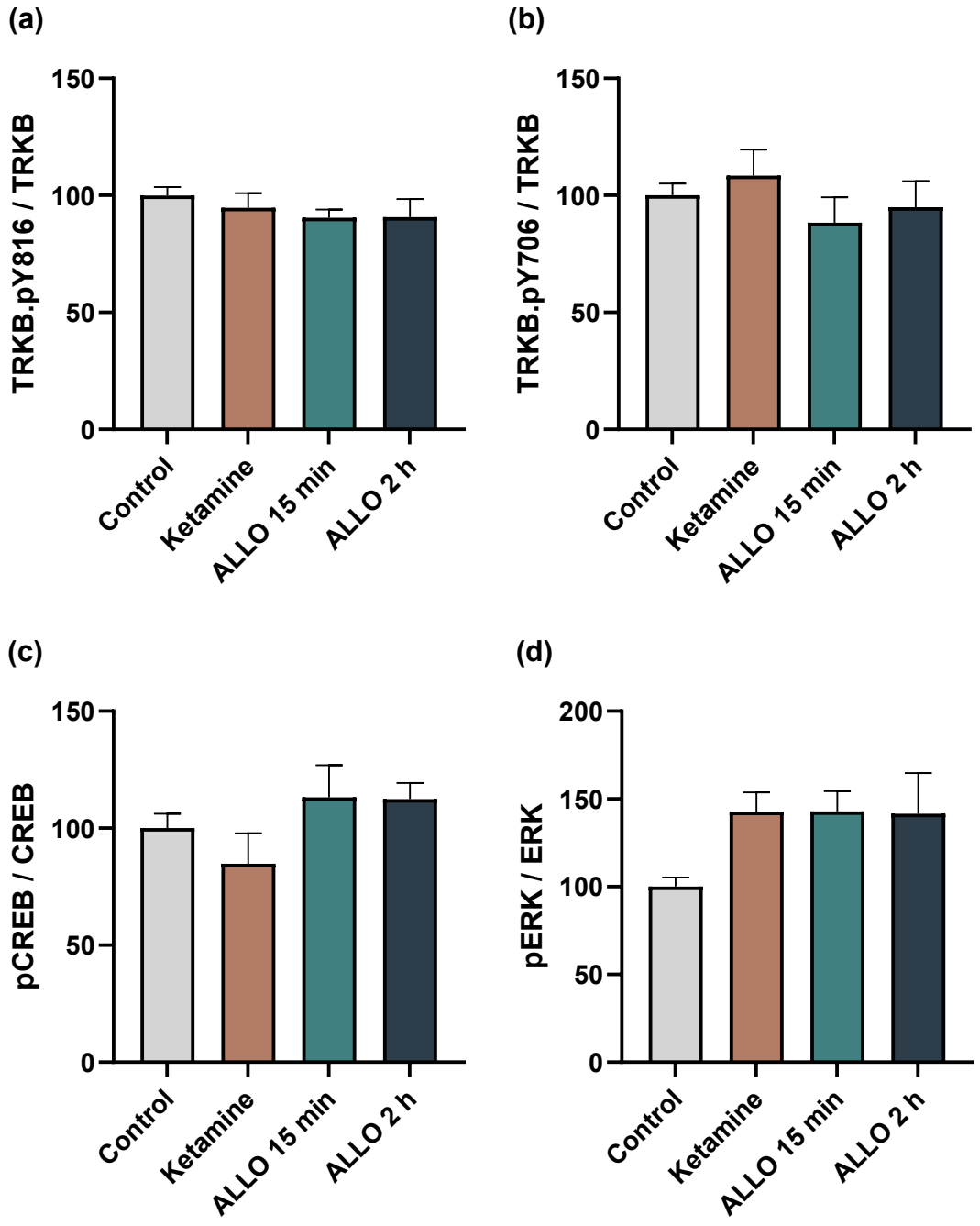

***Figure 9*. ALLO does not increase phosphorylation of TRKB signalling proteins in transfected N2a cells**

N2a cells transfected to express TRKB were treated with ketamine (10 uM/15 minutes) or ALLO (1 uM/15 minutes or 1 uM/2 hours) and the amount of phosphorylated TRKB, CREB and ERK determined by Western Blot (n = 6/group). (a) There was no effect of either ketamine or ALLO treatment on the phosphorylation of TRKB at Y816 residues. (b) There was no effect of either ketamine or ALLO on the phosphorylation of TRKB at Y706/707 residues. (c) There was no effect of either ketamine or ALLO on the phosphorylation of CREB. (d) There was no effect of either ketamine or ALLO on the phosphorylation of ERK. Data expressed as mean ± SEM of percentage from control.

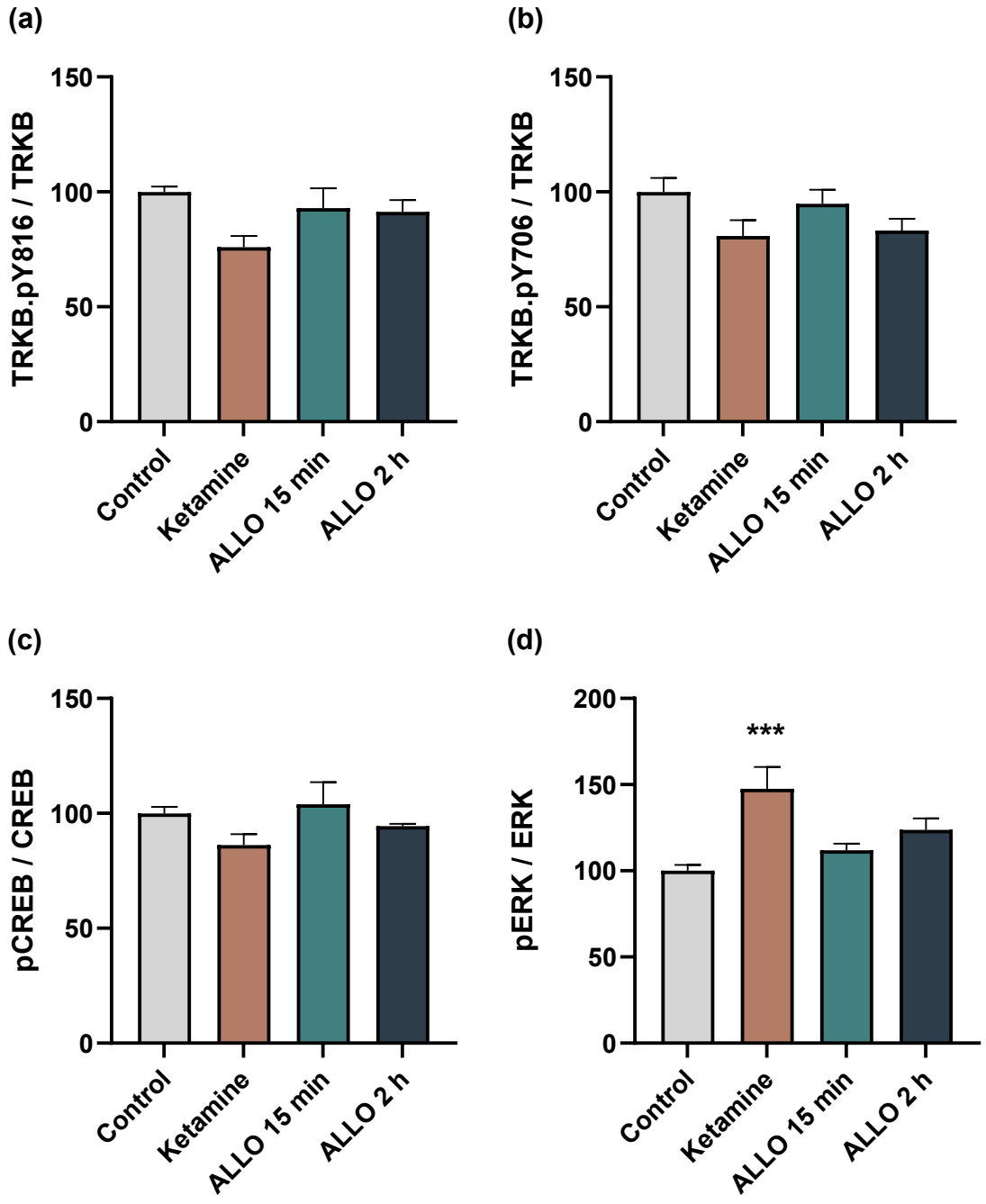

***Figure 10*. ALLO does not increase phosphorylation of TRKB signalling proteins in transfected HEK293T cells**

HEK293T cells transfected to express TRKB were treated with ketamine (10 uM/15 minutes) or ALLO (1 uM/15 minutes or 1 uM/2 hours) and the amount of phosphorylated TRKB, CREB and ERK determined by Western Blot (n = 6/group). (a) Treatment with ketamine showed a decrease in TRKB phosphorylation at Y816 residues [treatment: $F(3, 20) = 3.158$, $p = 0.047$]. (b) Both ALLO and ketamine treatment had no effect on TRKB phosphorylation at Y706/707 residues. (c) There was no increase in the phosphorylation of CREB following ALLO or ketamine treatment. (d) Ketamine treatment increased phosphorylation of ERK [treatment: $F(3, 20) = 7.149$, $p = 0.0019$]. Data expressed as mean ± SEM of percentage from control. *$p<0.05$, ***$p<0.001$ from the control (untreated) group, one-way ANOVA followed by Dunnett's *post hoc.*

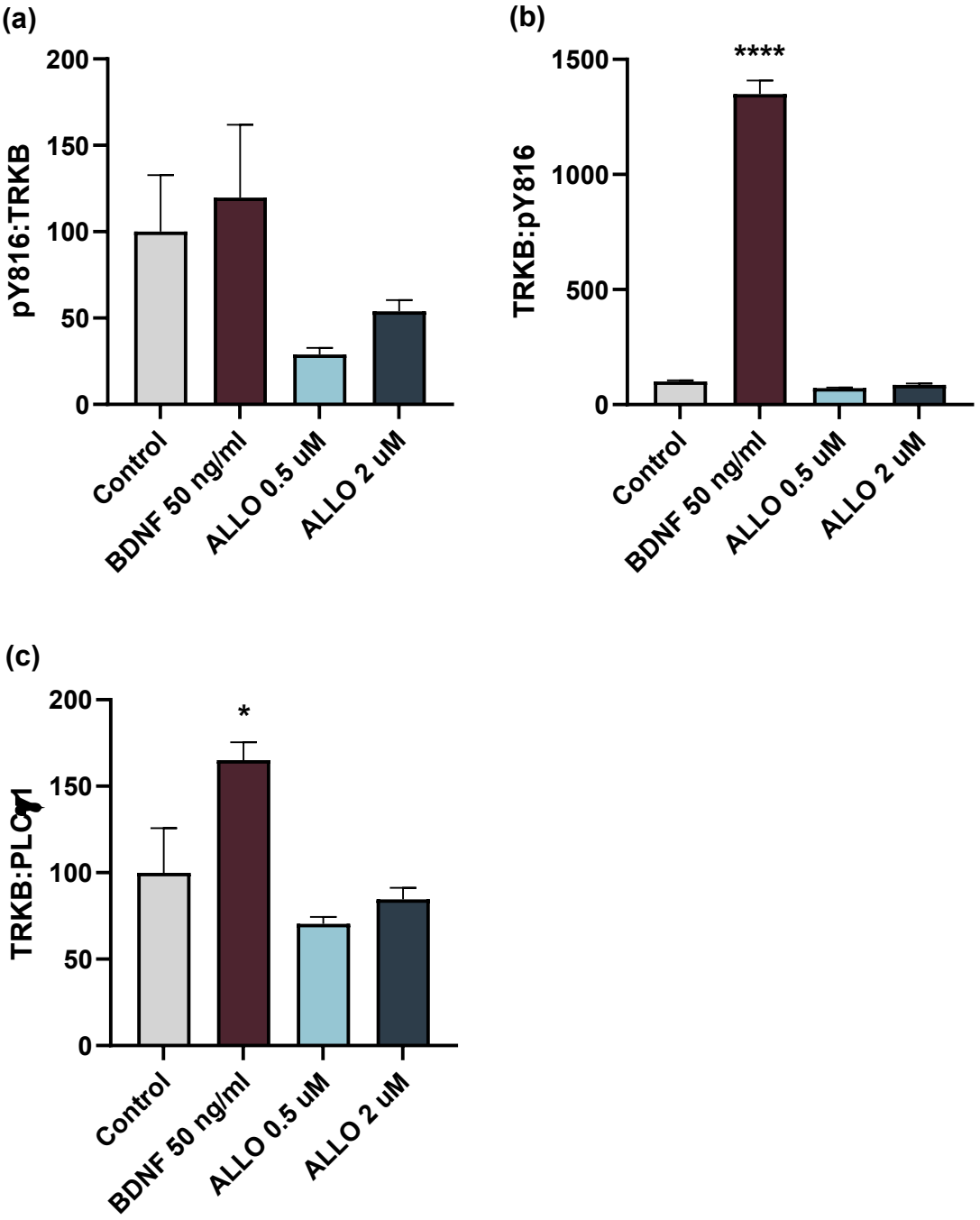

Figure 11*. ALLO does not increase TRKB phosphorylation *in vitro
E18 rat primary cortical neuronal cultures were treated with BDNF or ALLO and the amount of phosphorylated Y816 residues or PLCγ1 associated with TRKB was determined by sandwich ELISA. (a) Treatment with either BDNF or ALLO had no effect on TRKB phosphorylation at Y816 residues when the capture antibody is against pY816 (n = 3/group). (b) Treatment with BDNF showed increased TRKB phosphorylation at Y816 residues when the capture antibody is against TRKB [treatment: $F(3, 20) = 446.9$, $p<0.0001$], while ALLO treatment had no effect (n = 6/group) (c) Treatment with BDNF showed increased TRKB:PLCγ1 interaction [treatment: $F(3, 8) = 8.401$, $p = 0.0074$], while ALLO treatment had no effect (n = 3/group). Data expressed as mean ± SEM of percentage from control. *p<0.05, ****p<0.0001 of control (vehicle) condition, one-way ANOVA followed by Dunnett's *post hoc.*

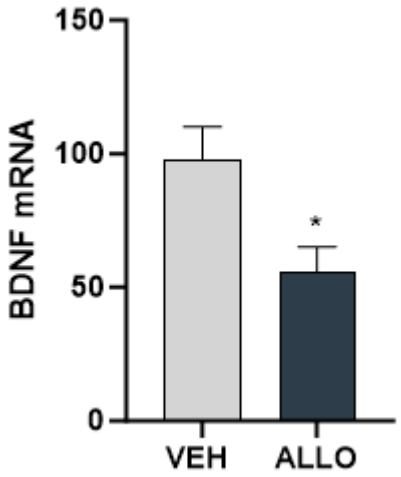

***Figure 12*. BDNF mRNA decreases after 30 minutes of ALLO treatment**
E18 rat primary cortical neuronal cultures were treated on DIV 10 with 2 μM ALLO for 30 minutes or the vehicle DMSO and the levels of BDNF mRNA determined by qPCR with HPRT as the housekeeping gene (n = 6/group). BDNF mRNA levels decrease following 30 minutes of treatment with ALLO [treatment: $t(8) = 2.884$, $p = 0.0204$]. Data expressed as mean ± SEM. *$p<0.05$, unpaired t-test.

Discussion

The approval of synthetic ALLO for the treatment of PPD solidified its antidepressant status, though the mechanism responsible for its mood alleviating effects has not been established. It is widely accepted that ALLO modulates $GABA_A$ receptors (Maguire & Mody, 2008), but whether this neurotransmitter effect is responsible for the therapeutic response is unclear. The present *in vitro* study contributes evidence in support of the hypothesis that ALLO potentiates TRKB dimerization in a manner that is akin to the positive allosteric modulation by other antidepressants.

4.1 TRKB dimerization

The dimerization of TRKB monomers upon BDNF binding is a critical first step in BDNF signalling as it initiates the autophosphorylation of TRKB's intracellular domains (Cunningham & Greene, 1998; McDonald et al., 1995). Most antidepressants act as positive allosteric modulators of BDNF signalling by directly binding to and stabilizing the TRKB dimer in a signalling competent conformation, potentiating BDNF signalling through increased TRKB dimerization (Casarotto et al., 2021). To investigate whether ALLO had similar effects on TRKB dimerization, I conducted PCAs in N2a cells and found that a 2 μM ALLO treatment increases TRKB dimerization. Considering that N2a cells do express small amounts of BDNF (Haapasalo et al., 1999), this increase in TRKB dimerization could be due to an allosteric effect that facilitates BDNF binding, which would align with the known mechanism of other antidepressants. To examine whether ALLO potentiates BDNF-induced TRKB dimerization, I repeated the experiment with increasing concentrations of BDNF with or without ALLO and found that ALLO increases TRKB dimerization at the highest concentration of BDNF (50 ng/ml). However, should ALLO act allosterically, we would have expected to see increased TRKB dimerization at lower BDNF concentrations in the ALLO condition, especially when compared to BDNF alone. Unfortunately, we failed to capture any significant dimerization in the BDNF-only condition, including at high BDNF concentrations where it was expected. It's possible that the absence of TRKB dimerization in the BDNF-only condition was due to BDNF functionality issues, which were ongoing in the lab; TRKB dimerization and phosphorylation in response to BDNF was inconsistent and higher than usual concentrations of BDNF were needed to produce them. Considering this, the diminished effect of BDNF could be limiting the

ability to capture a potentiating effect of ALLO on BDNF signalling and under typical conditions we would witness increased TRKB dimerization at lower concentrations of BDNF. Regardless, if ALLO potentiates BDNF signalling via an allosteric mechanism then its effect on TRKB dimerization should depend upon BDNF binding. Considering that BDNF binds to TRKB within its extracellular domain (Cunningham & Greene, 1998; McDonald et al., 1995), I investigated this by conducting PCAs with N2a cells transfected to express TRKB_ΔEC, a modified TRKB molecule which lacks the extracellular domain, expecting that ALLO would not increase TRKB dimerization since BDNF could not bind. As expected, neither BDNF nor ALLO had a significant effect on TRKB dimerization, further supporting the role of ALLO as an allosteric modulator of BDNF signalling.

Since ALLO's effect on TRKB dimerization appears to be similar to other antidepressants, we wondered whether it also interacts with the receptor at the same transmembrane domain region. Although the specific amino acid residues involved with antidepressant binding at the receptor differs slightly amongst antidepressants, some overlap exists with those identified as functionally important. Specifically, mutating the Y433 residue to a phenylalanine (Y433F) disrupts the allosteric effect of fluoxetine, ketamine (Casarotto et al., 2021), and LSD (Moliner et al., 2023). Should ALLO function similarly, we expected that the same Y433F mutation would also disrupt its effect on TRKB dimerization. To test this, I conducted PCAs in N2a cells transfected to express TRKB TRKB_Y433F and applied ALLO treatments. As expected, the Y433F mutation abolishes the effect of ALLO on TRKB dimerization, suggesting that ALLO's site of interaction at the receptor at least partially overlaps with other antidepressants.

4.1.1 Methodological considerations

For investigating TRKB dimerization, we chose PCA as our protein-protein interaction assay for several reasons. To start, PCA is conducted in live cells which represents a more biologically relevant environment than that of isolated proteins. Second, the split-luciferase interaction is reversible and ensures that the measured luminescence reflects the number of dimerized TRKB monomers at that moment, as opposed to for example a GFP interaction which locks once it has refolded into its functional state. Third, PCA is highly customizable with regards to the plasmid used for transfection,

allowing us to alter the receptor and address how the loss of the extracellular domain (TRKB_ΔEC) or the mutation of a key residue involved in antidepressant binding (TRKB_Y433F) affects TRKB dimerization in the presence of ALLO. Additionally, PCA yields a high degree of sensitivity, is high throughput, and cost-effective compared to other protein-protein assays. However, PCA is not without its drawbacks which should be taken into consideration when interpreting the results. Firstly, PCA requires a high N number as it is prone to variability from e.g. small pipetting errors leading to well volume discrepancies that affect final luminescence measurements. Secondly, transfection results in protein overexpression and does not represent a true physiological environment. In this case, the overexpression of TRKB results in a high level of basal dimerization, potentially creating a ceiling effect or masking more subtle impacts on TRKB dimerization. Lastly, PCA is unable to capture very short-lived dimerizations; the shortest time-point for obtaining measurements with PCA is 30 minutes to allow for cells to stabilize and decrease variability between wells.

4.2 TRKB phosphorylation and signalling

After TRKB dimerization, the autophosphorylation of tyrosine residues within the intracellular catalytic domains initiates 3 main signalling pathways: Ras-MAPK, PI3K-Akt, and PLCγ-Ca2+ (Kaplan & Miller, 2000; Minichiello, 2009). By increasing dimerization, both ketamine and fluoxetine potentiate the PLCγ-Ca2+ through increased phosphorylation of TRKB residue Y816 (Casarotto et al., 2021), which recruits PLCγ1 to the receptor and activates the pathway (Minichiello, 2009). If ALLO is also an allosteric modulator of BDNF signalling, we would expect to see a similar increase in TRKB phosphorylation and PLCγ signalling. To investigate this, I conducted Western Blot analysis of ALLO treated cells to determine phosphorylation of TRKB residues Y816 and Y706/707 (included as an extra test of TRKB phosphorylation) and downstream signalling proteins ERK and CREB. The results were compared to cells which were untreated or treated with ketamine, which was chosen as the positive control due to BDNF functionality issues and ketamine having been previously shown to increase Y816 phosphorylation. The experiment was repeated in 3 different cell lines: N2a, HEK293T, and MG87.TRKB. This was done to establish whether TRKB phosphorylation was affected by transfection or cell type, since both N2a and HEK293T cell lines were transfected to transiently overexpress TRKB while MG87.TRKB stably expresses TRKB. Unfortunately, both ketamine and

ALLO failed to increase TRKB phosphorylation in all cell lines. Between the transiently transfected N2a and HEK293T cells, the only measured effect was an increase in ERK phosphorylation following ketamine treatment in HEK293T cells. However, both ALLO and ketamine treatment increase CREB phosphorylation in the continuously TRKB expressing MG87.TRKB cells. Since transient transfection leads to overexpression of TRKB and a higher level of basal dimerization, it is possible that this creates a ceiling effect, masking the effect of drug treatments on protein phosphorylation in N2a and HEK293T cells. This would help explain why the effect of ALLO on CREB phosphorylation is only seen in the MG87.TRKB cells, which do not overexpress TRKB to the same degree. However, this does not explain why we do not see increased TRKB or ERK phosphorylation. We expected TRKB phosphorylation to still be present after 15 minutes, but it is possible that signalling is faster than expected. Additionally, since CREB is a transcription factor at the end of the pathway any correlation with TRKB activation is indirect. However, other well-characterized antidepressants, such as fluoxetine, fail to induce TRKB phosphorylation *in vitro*, while still being able to affect the downstream signalling pathways and TRKB interactome (Fred et al., 2022; Fred et al., 2019). Therefore, it is not entirely surprising that other compounds that promote TRKB activity exhibit a similar behaviour. *In vivo* studies will clarify this aspect that remains unsolved.

4.2.1 ELISA

I chose to use Western Blot for this initial investigation into protein phosphorylation as it allows for the analysis of multiple proteins within the same sample and is otherwise a well-established and reliable method. However, it is not a very sensitive assay and if you do see an effect, it is typically a very big effect. Considering this, I conducted additional ELISA experiments using neurons treated with ALLO or BDNF and applied a sandwich technique to measure Y816 phosphorylation and PLCγ1 recruitment. ELISA was not initially chosen since it is an expensive assay and requires a high N due to its susceptibility to pipetting bias. However, since ELISA is much more sensitive than Western Blot and neurons reflect a more biologically relevant environment without TRKB overexpression, we expected to see an increase in Y816 phosphorylation and PLCγ1 recruitment following ALLO treatment. Unfortunately, preliminary results show that ALLO does not increase Y816 phosphorylation and PLCγ1 recruitment. However, the same challenge in capturing antidepressant effects

on TRKB phosphorylation *in vitro* that was mentioned for Western Blot also applies here. Of note, BDNF did successfully increase Y816 phosphorylation and PLCγ1 recruitment, but this effect was only seen when TRKB was used as the capture antibody rather than pY816. This suggests that the order of antibodies used should be considered in future experiments utilizing this sandwich ELISA technique for TRKB phosphorylation.

4.3 BDNF mRNA

The final stages of BDNF signalling cascades results in the activation of transcription factors with genomic effects that include the transcription of BDNF mRNA (Yasuda et al., 2007). As such, potentiated BDNF signalling leads to an upregulation of BDNF mRNA expression, which occurs following chronic fluoxetine treatment (Molteni et al., 2006; Musazzi et al., 2009). Should ALLO similarly potentiate BDNF signalling, we expected that there would be an upregulation of BDNF mRNA following treatment. To test this, primary cortical neurons were treated with ALLO and the BDNF mRNA was extracted and measured using qPCR. Unexpectedly, there was a decrease in BDNF mRNA after 30 minutes of ALLO treatment. However, this result does align with one study which saw that hippocampus BDNF content first decreased by 25% 30 minutes after intraperitoneal injection of ALLO in male rats, before significantly and transiently increasing at 180 minutes post-injection (Naert et al., 2007). The relationship between antidepressants and BDNF mRNA transcription and translation appear to be complex, with one study showing that BDNF mRNA and protein levels during antidepressant treatments did not correlate, and the induction of mature protein preceded that of mRNA (Musazzi et al., 2009). Therefore, it is possible that the decrease in BDNF mRNA is due to an increase in protein translation during the initial response, with increased mRNA transcription appearing later. Considering this, the experiment would benefit from being repeated with longer treatment timepoints, as it is likely that increased mRNA production occurs later.

4.4 Study limitations and future directions

The present study has certain limitations which are in addition to the methodological ones outlined in each section. Firstly, the *in vitro* nature of the study limits the translatability of the results. Cultured primary neurons lack many *in situ* components and likely do not reflect an intact nervous system, while the use of non-neural cell lines

even further limits our ability to interpret the results. Secondly, the unresolved BDNF functionality issues in the lab required the use of unusually high concentrations of BDNF with inconsistent outcomes and challenges the reliability of the experimental results. Thirdly, complex pharmacokinetics greatly influence how any administered drug affects the nervous system and the direct application of ALLO into cell culture media is not reflective of this process. Consequently, potential differences in metabolism and distribution of ALLO could affect how it impacts BDNF-TRKB signalling. Despite these limitations this study provides direction for future experiments; next steps are to conduct *in vivo* studies to investigate the effect of ALLO on TRKB phosphorylation/BDNF signalling.

Conclusion

The results of this study provide evidence in support of ALLO acting as a positive allosteric modulator of BDNF signalling (Fig. 13). Like other antidepressants, it appears to increase TRKB dimerization in a BDNF-dependent manner. Additionally, the Y433 residue in the transmembrane domain of TRKB, which is essential for the iPlasticity effect of antidepressants, also appears to be essential for ALLO's effect on TRKB dimerization, suggesting a potentially overlapping interaction site. As such, we propose in addition to alterations in $GABA_A$ signalling, an increase in neuroplasticity via positive allosteric modulation of BDNF signalling may also be involved in the therapeutic effect of ALLO.

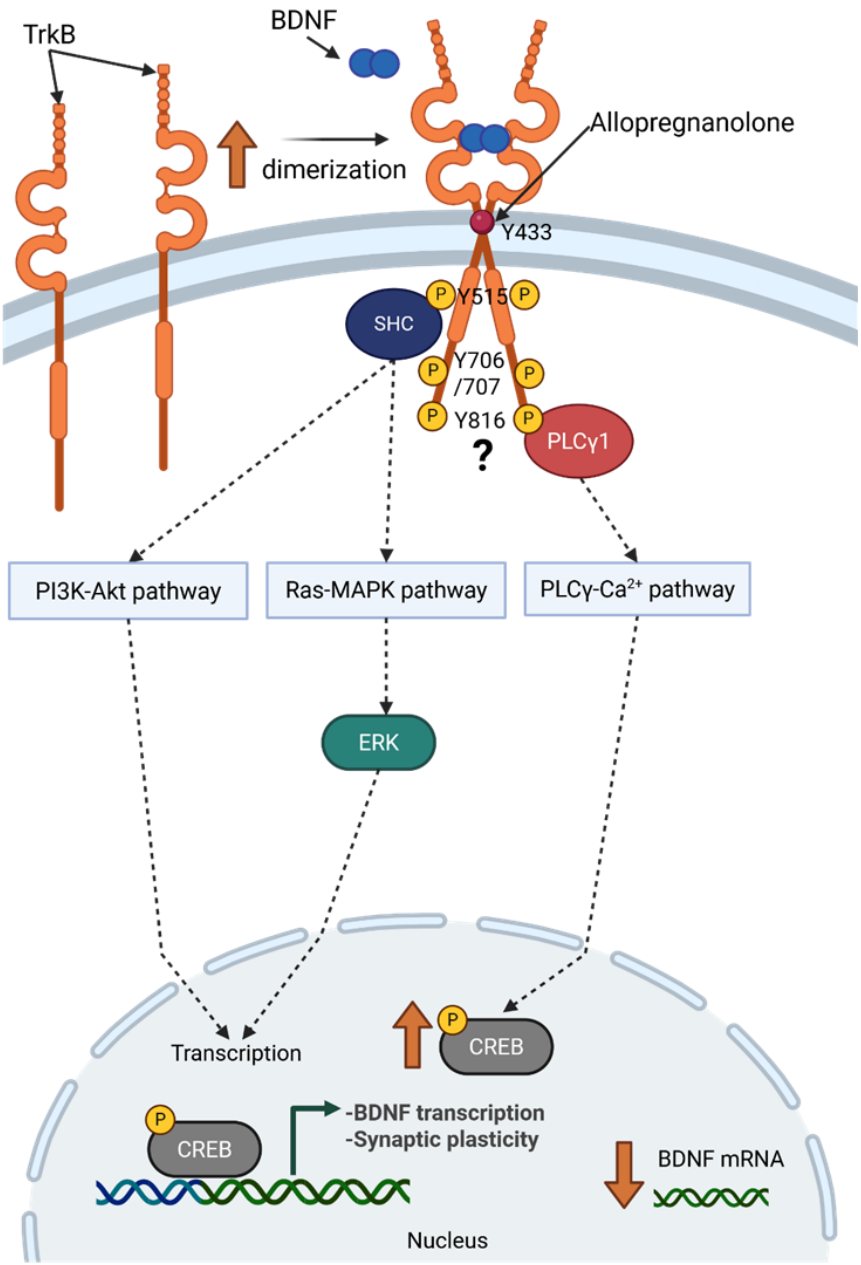

***Figure 13*. Study results regarding the effect of ALLO on BDNF signalling**
BDNF binds to the extracellular domain of TRKB, initiating the dimerization of TRKB monomers and the subsequent phosphorylation of tyrosine residues within the intracellular domain (Cunningham & Greene, 1998; McDonald et al., 1995). ALLO potentiates TRKB dimerization in a manner that is dependent upon BDNF binding, and the Y433 residue within the transmembrane domain of TRKB is required for this effect. The double tyrosine residues Y706/707 within the autophosphorylation domain are necessary for TRKB activation (Saarelainen et al., 2003), while Y515 and Y816 act as docking sites for signalling molecules (Minichiello, 2009). At Y515, SHC adaptor molecules are recruited and initiate the PI3K-Akt and Ras-MAPK signalling pathways, while Y816 recruits PLCγ1, activating the PLCγ-Ca^{2+} pathway (Minichiello, 2009). This study was unable to capture any effect of ALLO on Y706/707 and Y816 phosphorylation, or PLCγ1 recruitment, and Y515 was not measured. This study was also unable to capture any effect of ALLO on ERK phosphorylation as part of the Ras-MAPK pathway. Within the PLCγ-Ca^{2+} pathway, ALLO increases phosphorylation of CREB. CREB phosphorylation initiates gene transcription related to synaptic plasticity (Minichiello, 2009), including BDNF mRNA production (Yasuda et al., 2007). However, a 30-minute ALLO treatment decreases total BDNF mRNA. Created with BioRender.com.

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