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EXPLORING THE MOLECULAR MECHANISMS OF THE HUMAN VGF-DERIVED ANTIDEPRESSANT NEUROPEPTIDE TLQP-62 ON NEURODIFFERENTIATION

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"Cada pessoa deve trabalhar para o seu aperfeiçoamento e, ao mesmo tempo, participar na responsabilidade colectiva por toda a humanidade"

"Every person must work for his own improvement, and at the same time he must share a general responsibility for all humanity."

Marie Skłodowska Curie

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Aos meus pais:

Por fazerem de mim quem sou.

Por estarem sempre presentes.

Por me apoiarem e acreditarem em mim.

Mesmo quando eu não o faço.

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Thank you

RESUMO

SUMMARY

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RESUMO

As enfermidades neuropsiquiátricas afectan un 10% da población mundial. Porén, do ponto de vista biolóxico e molecular non hai ainda moita información que permita un tratamento e diagnóstico efectivos. Un dos procesos que está afectado nestas enfermidades é a neuroxenese. Varios xenes e as correspondentes proteínas ten sido implicados en enfermidades mentais e neurolóxicas, e na neuroxénese adulta, como é o caso de DISC1 (do ingles, *Disrupted in Schizophrenia 1*) e VGF (sen acronimo) que estan implicados na via de sinalización BDNF/TrkB/ /CREB da neuroxénese hipocampal. DISC1 regula VGF via PI3K/AKT /CREB na liña celular humana SH-SY5Y. VGF é inducida por NGF e BDNF, e é o precusor de vários outros pequenos neuropéptidos, como TLQP-62. Este péptido ten un efeito antidepresivo promovendo a neuroxénese hipocampal aumentando a actividade sináptica e dendritoxénese, sendo un alvo de investigación interesante na busca de tratamento para estas enfermidades.

No presente estudo, dividido en tres capítulos, se evaluaron os efeitos de TLQP-62 humano na diferenciación da liña celular SH-SY5Y, se intentou a encontrar un receptor para TLQP-62 e aínda se determinou a estrutura tridimensional deste péptido.

TLQP-62 ten a capaciade para inducir a diferenciación das células promovendo o aumento das neuritas. No estudo proteómico, TLQP-62 induce a expresión de varias proteinas relacionadas con procesos de neurodesenvolvimento e plasticidade sináptica, como crecimento e comunicación celular, metabolismo e biosíntese, función mitocondrial e resposta imunolóxica. Estes procesos son fundamentais para a neuroxéneses e estan desregulados en varias enfermidades mentais e neurolóxicas.

Para mellor entender os mecanismos moleculares de TLQP-62 na neuroxénese e neurodiferenciación é necesario determinar o receptor de TLQP-62. Utilizouse cromatografia de afinidade pola avidina para isolar o receptor do lisado de celulas SH-SY5Y con biotina-TLQP-62, identificándose o receptor acoplado á proteina G OR5P3. Comprobouse a capacidade de TLQP-62 para aumentar os niveis de cAMP num modelo celular que sobreexpresa OR5P3. A chaperona HSPA8 foi también identificada e a sua capacidade para interaccionar con TLQP-62 foi evaluada por análise de redistribuición dinámica de masa.

A estrutura de TLQP-62 foi avaliada e determinouse ser maioritariamente desorganizada en transición con unha alfa-helice, que probabelmente será estabilizada en complexo con HSPA8 ou OR5P3.

Futuros estudos estruturais e moleculares son necesarios para mellor comprender os mecanismos de acción de TLQP-62 e de que forma é que un potencial agonista pode ser producido e usado como tratamento para algumas enfermidades neuropsiquiátricas e neurolóxicas.

SUMMARY

Neuropsychiatric disorders, as major depression, schizophrenia or bipolar disorder, affect about 10% of world population, having a huge impact on society and a bigger stigma. Knowledge from a molecular and biological view is still poor, and diagnostic and treatment are lacking effectiveness, leading to a high suicide index.

These disorders have a genetic and environmental component, and some genes have been pointed out as possible risk factors. Although diagnose is performed through self-reporting of patient in a clinical interview and behaviour observation, there are evidence from patients suffering from these disorders of hippocampal abnormality with reduced volume and decreased cell proliferation, which affect cognitive functions and memory. Changes can be reversed by treatment with antidepressants, antipsychotics or physical exercise, by having an impact on neurogenesis.

Neurogenesis is the generation of new neurons from neural stem cells, occurring mainly during the embryonic brain development, but it continues during life in some areas of the brain, mainly being in the subgranular zone of dentate granule cells in the *dentate gyrus* of the hippocampus and subventricular zone of interneurons in the olfactory bulb. This process generates new cells that suffer migration, differentiation with axon and dendrite outgrowth, and synapse formation to integrate the pre-existing neuronal circuit, confering plasticity to those regions. Understanding the molecular mechanisms underlying adult neurogenesis may give critical insight for successful treatment for these disorders. The BDNF/TrkB/CREB signalling pathway has been shown to be implicated in hippocampal neurogenesis, together with some other proteins, as DISC1 and VGF.

DISC1 was found mutated, leading to its dysregulation, in individuals with schizophrenia, schizoaffective disorder, bipolar disorder or major depression. This protein is linked to neurogenesis and it functions as a scaffold protein, interacting with several other proteins important for neurodevelopment and synaptic function. It has been previously reported that DISC1 knockdown produces a significant downregulation of VGF, a nerve growth factor responsive gene, in SH-SY5Y cells. DISC1 apparently does not interact directly with VGF but seems to regulate this gene expression through the PI3K/AKT/CREB pathway.

VGF is a peptide precursor for TLQP-62, an antidepressant neuropeptide that promotes hippocampal neurogenesis, with an effect on memory and learning, through a BDNF-dependent mechanism, increasing synaptic activity and dendritic branching. Thus, this neuropeptide is a very attractive target for further search and investigation of its role and molecular mechanisms in neurogenesis and neurodifferentiation. Also, the identification of TLQP-62 receptor(s) and mediated signalling pathways are crucial for a better understanding of these neuropeptide molecular mechanisms, as well as the three-dimensional structures, for further investigation for agonists to be used as a treatment for chronic mental disorders.

The human derived cell line SH-SY5Y reproduces biochemical and morphological properties of neurons, being often used as a human *in vitro* model. Those cells can be induced to differentiate into a more neuron-like phenotype, through retinoic acid induction, and express high levels of VGF. Thus, those cells can be used as a model for neurogenesis concerning the neurodifferentiation step.

In the present study the effect of TLQP-62 on SH-S5Y5 was evaluated, concerning its ability to induce proliferation and/or neurodifferentiation with neuritogenesis and dendritogenesis. Morphological and proteomic evaluation were performed. TLQP-62 is capable alone of induce neurodifferentiation on SH-SY5Y cells, rather than proliferation, promoting more and longer dendrites with occasional connection between cells. Moreover, in a proteomic analysis, several proteins involved in neurodevelopment and synaptic

plasticity processes – as cell growth and communication, biosynthesis and metabolism, fatty acid and glucose metabolism, and immune an inflammatory response – are increased by TLQP-62. These processes are known to be implicated in neurogenesis and in neurological disorders.

To better understand the molecular mechanisms of TLQP-62 in neurogenesis and neurodifferentiation processes, as dendritogenesis, it is crucial to know the TLQP-62 receptor(s), and for that an avidin affinity chromatography assay was performed to membrane fraction of SH-SY5Y cells incubated with biotin-TLQP-62 crosslinked with sulfo-EMCS. Olfactory receptor 5P3 was isolated, a GPCR, apparently classified as an olfactory receptor based on homology. TLQP-62 is capable of increase cAMP levels in a cell model overexpressing OR5P3, proving it is an active ligand for this receptor and may act through a Gs protein signalling pathway. HSPA8 and HSPD1 were also isolated. HSPA8 have been previously described as a TLQP-21 binding protein, and in the present study was proved to interact with TLQP-62 by label-free dynamic mass redistribution analysis.

The structure of TLQP-62 was also explored in order to further understand its interaction with OR5P3 and HSPA8. In solution TLQP-62 acts as a random coil transitory with an α-helix, which might be stabilized upon binding to HSPA8 or to its receptor OR5P3. Moreover, HSPA8-OR5P3-TLQP-62 might form a complex to activate a signal transduction pathway. Further structural insights into this putative complex can help developing a pharmacological drug agonist for this receptor to enhance neurogenesis and to be used as a treatment of some neuropsychiatric and other neurological disorders.

All data taken together contribute to better understand TLQP-62 mechanism of action in neurogenesis and neurodifferentiation, and also in mental and neurological disorders.

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LIST OF ABBREVIATIONS

2D-DIGE	Dimensional fluorescence difference gel electrophoresis
5-HT	Serotonin
5-HTR	Serotonin receptor
5-HTT	Serotonin transporter
ACN	Acetonitrile
ADHD	Attention deficit hyperactivity disorder
AKT	Protein Kinase B
AMPK	AMP-activated protein kinase
ANK3	Ankyrin-G
APOE	Apolipoprotein
APP	Amyloid precursor protein
APS	Ammonium persulfate
ATF4/5	Activating transcription factor 4/5
BAI3	Brain angiogenesis Inhibitor 3
BDNF	Brain Derived Neurotrophic Factor
BPD	Bipolar disorder
BSA	Bovine serum albumine
CA	*Cornu ammoris*
CACNA	L-type voltage-gated calcium channel subunits
CaMKII	Calcium/calmodulin kinase II
cAMP	Cyclic adenosine monophosphate
CDK1	Cyclin-dependent kinase 1
CDK5	Cyclin-dependent kinase 5
CHAPS	3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate
CHD5	Chromodomain-helicase DNA binding protein 5
COMT	Catechol-O-methyltransferase
CRE	Cyclic adenosine monophosphate-responsive element
CREB	cAMP-responsive element-binding protein
DAO	D-amino acid oxidase

DAOA	D-amino acid oxidase activator
DAPI	4′,6-diamidino-2-phenylindole
DAT1	Dopamine transporter
DG	*Dentate girus*
DISC1	Disrupted in Schizophrenia 1
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DRD	Dopamine receptor
DTT	Dithiothreitol
EGTA	Egtazic acid
ER	Endosplasmic reticulum
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FEZ1	Fasciculation and elongation protein zeta-1
FSH	Follicle stimulating hormone
GABA	γ-aminobutyric acid
GNB3	Guanine nucleotide binding protein
GnRH	Gonadotropin realing hormone
Grb2	Growth factor receptor-bound protein 2
GPCR	G-protein coupled receptor
GRK3	G-protein receptor kinase 3
GSK3β	G lycogen synthase kinase-3 beta
GWAS	Genome-wide associated studies
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPA	Hypothalamic-Pituitary-Adrenal axis
IEF	Isoelectrofocusing
IgG	Immunoglobulin G
IPTG	Isopropyl β-D-1-thipgalactopyranoside
LIS1	Platelet-activating factor acetylhydrolase 1B1 (PAFAH1B1)
LH	Luteinizing hormone
MAOA	Monoamine oxidase A
MAP	Microtubule-associated Protein
MDD	Major Depressive Disorder

MEK	Mitogen-activated protein kinase kinase
mGluR	Metabotropic glutamate receptor
MIPT3	TRAF3-interacting protein 1
MTHFR	Methylene Tetradrofolate Reductase
mTOR	Mammalian target of rapamycin
NaCl	Sodium chloride
NCAN	Neurocan
NDEL1	Nuclear distribution protein nude like 1
Nf-kB	Nuclear factor-kB
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NMR	Nuclear magnetic resonance
NPAS3	Neuronal PAS domain protein 3
NPC	Neuronal progenitor cell
NRG1	Neuroregulin 1
NT3	Neurotrophin-3
PAGE	Polyacrylamide gel electrophoresis
PAK1	P21- activated kinase 1
PBS	Phosphate buffered saline
PCM1	Pericentriolar material 1 protein
PDE4	cAMP-specific 3',5'-cAMP phosphodiesterase 4
PDK	Phosphoinositide-dependent kinase 1
PKA	Protein kinase A
PKC	Protein kinase C
PKD	Protein kinase D
PI3K	Phoshphoinositide 3-kinase
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl fluoride
PROH	Proline dehydrogenase
PVDF	Polyvinylidene difluoride
RA	Retinoic acid
RAR	Retinoic acid receptor
RGS4	Regulator of G-protein signaling 4
ROS	Reactive oxygen species

RXR	Retinoid X receptor
S6K1	Ribosomal protein S6 kinase 1
SCZD	Schizophrenia
SDS	Sodium dodecyl sulfate
shRNA	Short hairpin ribonucleic acid
sulfo-EMCS	Sulfo-N-[ε-maleimidocaproyloxy]succinimide ester
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TCEP	Tris(2-carboxyethyl)phosphine
TPH	Tryptophan hydroxylase
Trk	Tyrosine kinase
TUBB	Tubulin beta chain
VEGF	Vascular endothelial growth factor

GENERAL

INTRODUCTION

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1 NEUROPSYCHIATRIC DISORDERS

Neuropsychiatric disorders, such as schizophrenia, major depression, bipolar disorder, and other affective disorders, are characterized as the impairment of intellectual functions and memory with deterioration of personality with the course of being progressive, stationary or reversible. In another words, neuropsychiatric illness causes brain dysfunction affecting perception, thinking, language, mood and behavior.

Neuropsychiatric disorders are the most prevalent neurological disorders affecting about 10% of world population.

Data obtained from US National Institute for Mental Health (NIMH) indicated that in 2012, 1.1% of adult US population was affected with schizophrenia, 2.6% by bipolar disorder and 6.9% by major depression (www.nimh.nih.gov). In European Union, the statistics indicate that 27% of adult population had experienced at least one episode of mental disorder, being major depression the leading cause of chronic disorders in Europe (www.euro.who.int). Mental disorders have a huge impact on society at a medical and financial level, being the third most costly medical condition around the world. Also, the lack of information and effective treatment makes these disorders a target of stigma on society. These progressive diseases are poorly understood from a molecular and biological point of view and are diagnosed as mere clinical phenotypes by self-reporting of patients in a clinical interview according to internationally defined criteria *(American Psychiatric Association APA. Diagnostic and Statistical Manual IV (DSM IV), Washington DC 1994).*

Effective treatment includes a combination of several factors as medication, healthy life style, education, job, peer support and psychotherapy. Although there are several antipsychotics, anti-depressants and mood stabilizing drugs, ~30% of patients do not respond to these drugs, have undesired secondary symptoms, or a poor efficacy, ameliorating some of the symptoms, but not the core pathology, like in the case of classical antipsychotic for schizophrenia, that prevent positive symptoms, but have limited efficacy for negative and cognitive symptoms (Figure GI.1). This can lead to a higher suicide index and thus better treatment is needed.

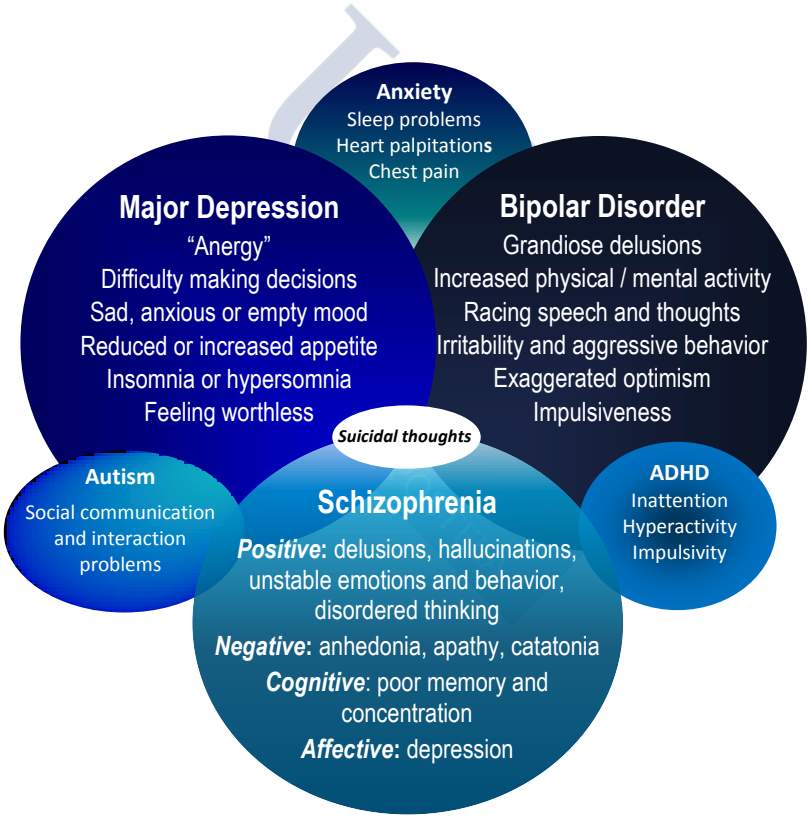

FIGURE GI.1 | SCHEMATIC REPRESENTATION OF 5 MAJOR MENTAL DISORDERS MAIN SYMPTOMS: schizophrenia, bipolar disorder, major depression, autism spectrum disorder and attention deficit hyperactivity disorder.

These disorders tend to run in families as they share certain genetic roots and symptoms, suggesting similarities at a biological level. The international research Psychiatric Genomics consortium conducted an analysis that incorporated data from the genome-wide associated studies (GWAS) of the 5 major mental disorders. The analysis revealed two L-type voltage-gated calcium channel subunits *CACNA1C* and *CACNB2* genes to be linked to the 5 disorders[1]. Another GWAS revealed that schizophrenia, depression and bipolar disorder are not only linked through shared genes, but the genetic mutations associated with them also work together to rule immunity, neuronal signaling and genome function later in life, which could mean that only one drug could be required to treat all these disorders[2].

The 5 major neuropsychiatric disorders are schizophrenia, bipolar disorder, major depression, autism spectrum disorder and attention deficit hyperactivity disorder (ADHD).

1.1 MAJOR DEPRESSION

According to NIMH and WHO, major depressive disorder (MDD; OMIM#608516) is the leading cause of disability worldwide and it can cause the affected person to suffer severely, compromising its normal social functions and activities, and leading, at its worse, to suicide. This common illness can be defined as the presence of a depressed mood or loss of interest or pleasure, and at least four other symptoms reflecting change in functioning, such as problems with sleep, eating, energy, concentration, and having feelings of guilt or low self-worth, for two weeks or longer. Many people that suffer from depression also suffer from anxiety symptoms.

Antidepressant drug treatments for both depressive and anxiety disorders target monoaminergic neurotransmission, based on serotonin and catecholamine hypotheses[3–5]. These hypotheses consist in the notion that diminished activity of serotonin and monoamines plays a causal role in the pathophysiology of depression that can be reversed by antidepressants, restoring normal functioning[6]. Selective serotonin reuptake inhibitors are used to treat depression and anxiety, by increasing hippocampal cell proliferation (neurogenesis) and enhanced expression of neuroplasticity related proteins, such as brain derived neurotrophic factor (BDNF). However not all patients benefit from it

MDD is one of the most common mental disorders affecting people from all ages.

and several drug devoid of major effects on serotonergic neurotransmission are effective to improve mood[7,8]. Thus, there is a growing consensus that altered monoaminergic transmission is insufficient to explain the etiology of depressive disorders and that currently used antidepressants instead are modulating other neurochemical systems that have a more fundamental role in MDD[9]. Another hypothesis is explained by Hypothalamic-Pituitary-Adrenal (HPA) axis hyperactivity, that leads to stress and has been associated with neuropsychiatric disorders as it causes hippocampal volume reduction with dendritic process atrophy, decreased neurogenesis and neuroplasticity and neuronal losses[10,11]. MDD can be also explained converging genetic, epigenetic and stress-induced deficits in GABAergic transmission, as the therapeutic effects of currently used monoaminergic antidepressants involve downstream alterations in GABAergic transmission. Reduced brain concentration of γ-aminobutyric acid (GABA) and alterations in GABA receptors mediating GABAergic inhibition are observed in MDD patients. GABA plays a prominent role in the brain control of stress, the most important vulnerability factor in mood disorders, controlling hippocampal neurogenesis and neural maturation[12].

It is known that no single gene is necessary and sufficient for MDD, that each susceptibility gene contributes a small fraction of the total genetic risk and that there is a complex genetic heterogeneity that can predispose individuals to similar syndromes that are clinically indistinguishable. Several genetic biomarker candidates have been proposed for better understanding and treatment of depression, although these are dependent of environmental factors[13]. Some of those genes that have been pointed out as risk factors for MDD are *CACNA1C*[1], Serotonin Transporter (*5HTT*)[14,15], Serotonin Receptors (*5HTR*)[16–21], Catechol-O-methyltransferase (*COMT*)[22,23], Dopamine Transporter (*DAT1*)[24,25], Dopamine Receptors (*DRD*)[26–30], Tryptophan Hydroxylase (*TPH*)[31], Methylene Tetrahydrofolate Reductase (*MTHFR*)[13,32,33], Apolipoprotein E (*APOE*) [34,35], Guanine nucleotide binding protein (*GNB3*)[36,37], Cyclic adenosine monophosphate-responsive element-binding protein 1 (*CREB1*)[38–41], *BDNF*[42,43].

1.2 BIPOLAR AFFECTIVE DISORDER

Bipolar affective disorder (BPD; OMIM 125480), also known as major affective or manic-depressive disorder, is characterized by the shifting from a depression mood of feeling down, empty or hopeless, with lack of energy, to manic episodes, involving feeling "up", with a lot of energy and irritable mood, over activity, inflated self-esteem and a decreased need for sleep. Sometimes patients can suffer psychotic symptoms, as hallucinations or delusions, anxiety and ADHD[44]. Symptoms of ADHD include poor concentration, distractibility, impulsivity, restlessness, and agitation that are also features of a manic episode. Anxiety worsen the course and prognosis of BPD.

Some of the risk factor genes are common to BPD, SCZD and MDD.

Mood stabilizers, antidepressants and atypical antipsychotics help to treat bipolar disorder[45]. There is not a single hypothesis regarding genetic, biochemical, pharmacological or anatomical cause[46]. Biochemical investigations focus on neurotransmitters (serotonin, catecholamines, GABA, glutamate and others), hormones (brain-derived neurotrophic factor, thyroid and others), and steroids. The catecholamine hypothesis explains mania as an excess, and depression due to a depletion of catecholamines. Norepinephrine has been implicated because of abnormalities linked with depression including its modulation by tricyclic antidepressants. Dopamine has been implicated because the dopamine precursor L-dopa, amphetamines, and tricyclic antidepressants often produce hypomania in bipolar patients. Antipsychotic medications that selectively block dopamine receptors are effective against severe mania. The "permissive serotonin hypothesis" says that low serotonergic function accounts for both manic and depressive states through defective dampening of other neurotransmitters (mainly norepinephrine and dopamine). Some use this as an explanation as to why some bipolar patients do better on such antidepressants, including rare cases of mania that dissipate. Neuroanatomical and neuroimaging studies show that lesions in the frontal and temporal lobes are most frequently associated with bipolar disorder. Left-sided lesions tend to be associated with depression and right-sided lesions with mania. The circadian rhythm desynchronization has also been implicated in bipolar disorder.

There is evidence of heritability and specific genes have been linked to the predisposition to bipolar disorder[26,47–49]. Some of those are *CACNA1C*[1,50–52], Ankyrin-G (*ANK3*)[50,53–55], Neurocan (*NCAN*)[56–58], Monoamine oxidase A (*MAOA*)[48,59,60], *COMT*[61,62], Dysbindin[63], *MTHFR*[33,64,65], *5HTT*[26,66], *5HTR1A*[17], D-amino acid oxidase activator (*DAOA*)[59,67,68], *BDNF*[69–72], Nesprin[73], Disrupted in Schizophrenia 1 (*DISC1*)[74], G-protein receptor kinase 3 (*GRK3*)[75–77]. Some of these genes were also found to be linked to schizophrenia[49,61] and to MDD[73] (Table I.1). Although bipolar disorder and schizophrenia are classified and treated as separate diseases with separate etiologies, there are similar features and genetic observations that bring these two disorders closer, and some individuals show mood and psychotic features classified as schizoaffective disorder. These three disorders tend to occur in members of a same family[78].

1.3 SCHIZOPHRENIA

Schizophrenia (SCZD; OMIM#181500) is a chronic severe mental illness affecting thought, feeling and behaviour. It is not as common as depression, but the symptoms are very disabling. Children can have schizophrenia, but usually the first episode happens in the adolescence up to the age of thirty. Symptoms of schizophrenia can be positive, negative, cognitive and affective (Figure GI.1). Schizophrenia is a heterogeneous syndrome considered a disorder with a neuro-developmental component with a genetic inherited origin having a highly heritability. Many different genes may increase its risk in some cases (Table I.1) but not all, as environment can also have a strong influence on the onset of this disorder. So far, causes of the disease remain incompletely understood, but some risk factors have been identified[49]. Several candidate genes have been pointed out as having a role on schizophrenia aetiology: as *DISC1*[74,79–81], *NCAN*[56,58], *DRDs*[82–84], *COMT*[85,86], *MTHFR*[33,64], *5-HTR2A*[87], *BDNF*[88], Neuroregulin 1 (*NRG1*)[89–94], Dysbindin[95–97], D-amino acid oxidase (*DAO*) and *DAOA*[98–101], Regulator of G-protein signalling 4 (*RGS4*)[102–104], Proline dehydrogenase (*PRODH*)[105–107], Metabotropic glutamate receptor 3 (*mGluR3*)[108,109]. Some of these genes have also been associated with bipolar disorder and depression (Table GI.1)[110–112].

TABLE GI.1 | CANDIDATE GENES WITH THE STRONGEST EVIDENCE FOR A ROLE IN THE GENESIS OF SCHIZOPHRENIA, BIPOLAR DISORDER AND DEPRESSION (adapted from [49,113])

Gene	Hypothesized Role	SCZ evidence	BP evidence	MDD evidence
Disrupted in schizophrenia 1 (DISC1)	Poorly understood: possible roles suggested in synaptogenesis and neurodevelopment	+++	++	+
Neuroregulin 1 (NRG1)	Effects on synaptic plasticity, neuro-development and transmitter activity	++	+	
Dysbindin (DTNBP1)	Via synaptic glutamate release	++	+	
Regulator of G-protein signaling 4 (RGS4)	Modulates activity serotonergic and metabotropic glutamatergic receptors; modulated by dopaminergic transmission	+		
Dopamine receptors (DRD2/DRD3/DRD4)	Receptors for dopamine, associated with cognitive, emotional, and endocrine functions	+		++
Serotonin 1A/2A receptor (5-HTR1A/2A)	Receptor for serotonin, a neurotransmitter with many roles	+		++
Metabotropic glutamate receptor 3 (mGluR3)	NMDA receptor effects via affecting presynaptic glutamate release	+		
Proline dehydrogenase (PRODH)	Possibly affects ratio of L- to D-serine, that may have a role in regulation of NMDA receptor	+		
Catechol-O-methyl transferase (COMT)	Metabolizes cerebral monoamines including dopamine	+	+	+
D-amino acid oxidase activator (DAOA)	Modulation of synaptic transmission	++	+	+
Methylene tetrahydrofolate reductase (MTHFR)	Catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate	+	+	+
Brain-derived neurotrophic factor (BDNF)	Promotes survival and differentiation of selected neuronal populations of the peripheral and central nervous systems	+	++	+++

Schizophrenia is a neurodevelopmental disorder that affects neuronal dendritogenesis and synaptogenesis, and hippocampal abnormality is one of the most consistent findings in schizophrenia research, with decreased cell proliferation and reduced hippocampal volume, which is associated with impaired hippocampus functions, such as working memory, recognition memory, spatial pattern separation, and cognitive functions[114,115]. These changes of the hippocampus in those patients can be attenuated or even reversed by treatments with antidepressants, antipsychotics, or increasing physical exercise, by having an impact on hippocampal neurogenesis. The hippocampus is also implicated in bipolar disorder.

In the hippocampus, the *cornu ammonis* (CA) and the *dentate gyrus* (DG) regions are apparently the most affected in neurological disorders[116]. Postmortem studies have shown reduced neuronal number, size, density of GABAergic neurons, and dendritic arborization as well as evidence of glutamatergic dysfunction involving reduced N -methyl-D-aspartate (NMDA) and non-NMDA receptor expression[117–121]. Synaptic changes are also described and involve alterations in the expression of several synaptic proteins[122].

Genomic and genetic research contribute to major insights into the genetic basis, but the core of pathophysiology of these disorders still remains unknown. Predicted expression and function of the proteins related to the genetic data are limited by the post-translational modifications. Thus, several studies using proteomics have been performed in postmortem brain tissue from subjects with schizophrenia, bipolar disorder and major depression, showing alterations in cytoskeletal, synaptic, metabolic, immunological and mitochondrial proteins[123–127].

2 ADULT NEUROGENESIS AND NEUROPSYCHIATRIC DISORDERS

As most current treatments for neuropsychiatric disorders only ameliorate the most severe symptoms, it is critical to develop new therapeutic strategies that can target functional or anatomical domains of pathology, adult hippocampal neurogenesis is one of them[128]. Neurogenesis is the generation of new neurons from neural stem cells, occurring mainly during the embryonic brain development, but it continues during life in some areas of the brain, mainly being in the subgranular zone of dentate granule cells in the *DG* of the hippocampus and subventricular zone of interneurons in the olfactory bulb. This process generates new cells that suffer migration, axon and dendrite outgrowth and synapse formation to integrate the pre-existing neuronal circuits (dendritogenesis, axogenesis and synaptogenesis)[129].

Ault neurogenesis is the generation of new neurons from stem cells during life.

This is a unique form of structural and functional plasticity in the hippocampus and a brain region key to learning, memory and mood regulation. Thus, understanding molecular mechanisms that regulate adult neurogenesis may give critical insight into effector systems of successful treatments for psychiatric disorders and facilitate the development of more specific therapeutic strategies.

Hippocampal neurogenesis is involved in emotional regulation, memory and learning, and thus its impairment is not only associated with neuropsychiatric disorders, as major depression or schizophrenia, but also neurodegenerative disorders, as Parkinson‘s or Alzheimer’s disorders[130,131]. Constitutive neurogenesis in the hippocampus of the mature brain reflects plasticity and could be a potential target for modulation of a subset of cognitive and affective behaviours that are affected by multiple neuropsychiatric disorders.

Neurogenesis hypothesis has been proposed for major depression, bipolar disorder and schizophrenia due to the observation of patients' smaller hippocampus, indicating changes in number and properties of neural cells, as previously described[132]. Moreover, ventral hippocampus has been associated with modulation of affective behaviours and emotional memories, and dorsal hippocampus with spatial memory formation and the associative encoding of discrete stimuli[133]. Also, effectiveness of certain antidepressants is dependent of adult neurogenesis which is in turn increased by those antidepressants, anxiolytics and physical exercise reducing anxiety and depression behaviors[134,135]. Genetic manipulation of pathways that regulate neurogenesis has also been shown to interact with antidepressant treatment: deletion of BDNF receptor tyrosine kinase B (TrkB) results in decreased adult hippocampal neurogenesis, increased anxiety-like behaviour and inhibits antidepressants action[136,137]. Several other genes are involved in the regulation of neurogenesis, axogenesis, dendritogenesis and synaptogenesis, and in neuropsychiatric disorders, being interesting targets to better understand the molecular mechanisms of neurogenesis in neuropsychiatric disorders.

Wnt/glycogen synthase kinase-3 beta (GSK3β) signalling is known to have a role in neurogenesis during development and memory[138,139]. NMDA receptor has been proposed to control dendritogenesis of hippocampal neurons through calcium/calmodulin kinase II (CaMKII), phosphorylated extracellular signal-regulated kinase (pERK), phosphorylated CREB and histone 3[140]. Vascular endothelial growth factor (VEGF), an angiogenic factor, has also been shown to have a role in dendritogenesis[141] and the adhesion G-protein-coupled receptor (GPCR) Brain Angiogenesis Inhibitor 3 (BAI3) is critical for dendritogenesis and morphology regulation[142]. DISC1 is another protein involved in neurogenesis by interaction and regulation several others, including VGF (nonacronymic), a neuropeptide, also implicated in neurogenesis and memory[143–150].

3 DISRUPTED IN SCHIZOPHRENIA 1

Several independent genetic studies confirmed the relationship between *DISC1* and psychiatric disorders.

Although neuropsychiatric disorders have a major genetic component, few genes have been strongly implicated in these disorders, such as *DTNBP1* and *NRG1*. Another gene known as a risk factor is *Disrupted in Schizophrenia 1* which was identified at the breakpoint on chromosome 1 of the translocation (1;11) (q42.1;q14.3) that co-segregated in a large Scottish family with a wide spectrum of mental illness. Off note, DISC1 has also been linked to neurogenesis[146,147,151]. Among the carriers of the translocation, some were diagnosed with schizophrenia, bipolar disorder, and minor and major depression. In contrast, none of the non-translocation carriers have such a diagnosis. Linkage analysis showed a direct involvement of the translocation as a risk factor for the development of psychiatric illness[80,152–155]. Studies in Finnish population showed evidence for the involvement of the 1q32.2–q41 region of chromosome 1, proximal to the *DISC1*, in psychiatric disorders[79,156,157]. Studies in other populations including those of Taiwan, Britain, Iceland, and Scotland also supported linkage of chromosome 1q32-42 to major mental illness[50,158–162]. It was reported four *DISC1* haplotypes between exons 1 and 9 (HEP), associated with SCZD, schizoaffective disorder and BPAD, in the Finnish and North American populations (Figure GI.2)[74,163,164]. Association of SNPs in this gene with mental disorders have also been found in population from China[165], Scotland[166], Taiwan[167] and Japan[168].

Genetic studies also points to a relationship between DISC1 and traits as working memory, cognitive aging, decreasing grey matter volume in the prefrontal cortex, and abnormalities in hippocampus structure and function[169–173]. DISC1 binds several proteins involved in essential neuronal processes as neuronal migration and proliferation, neurite outgrowth, cytoskeletal modulation and signal transduction[174].

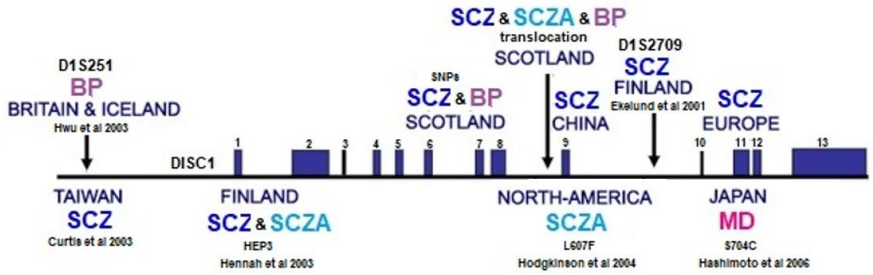

FIGURE GI.2 | SCHEMATIC REPRESENTATION OF *DISC1* GENE AND ITS GENETIC EVIDENCE IN PSYCHIATRIC DISORDERS IN WORLD POPULATIONS.

[Constructed from data of references in the image and Palo et al, 2007.]

3.1 GENE AND PROTEIN

Human *DISC1* gene (Ensembl ID: ENSG00000162946) is located at chromosome 1 in the q42 region overlapping with DISC2 open reading frame that might regulate DISC1 transcription[80]. *DISC1* is a large gene containing 13 exons over 414 kb of genomic DNA (Figure I.3). The full length transcript produced corresponds to 7.5 kb encoding a 854 amino acids protein. There have been identified different DISC1 isoforms at the RNA and protein level. Four DISC1 isoforms were identified indicating different functional roles[80,175]. However, more recently more than 50 DISC1 mRNA variants produced by alternative splicing were characterized[176].

DISC1 three-dimensional structure has not yet been solved to the date, mostly due to the difficulty to purify a fstable, correctly folded full length protein, as it tends to aggregate. Thus, the predicted structure is based on its sequence. Most secondary structure prediction programs indicate this protein is divided into a N-terminal mainly disordered region and a C-terminal helical region (Figure I.4)[80,177,178]. The N-terminal region (1 to 350 aa) is referred to as head domain and has a nuclear localization signal (NLS) and a serine-phenylalanine-rich motif[178]. The C-terminal region (351 to 854 aa) is predicted to have at least 4 coiled-coil regions and 5 regular alpha-helices. This region is responsible for the binding to a high number of proteins.

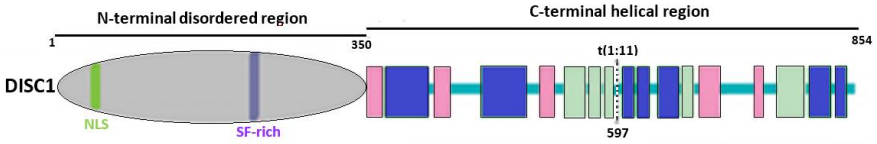

FIGURE GI.3 | SCHEMATIC REPRESENTATION OF *DISC1* PREDICTED SECONDARY STRUCTURE.
In pink, regular α-helices; in blue coiled-coil helices; light-green, ambiguous helix. Sequence motifs NLS and SF-rich, at the N-terminal head domain, and translocation break point t(1;11) at 597 residue at C-terminal coiled domain are shown.

[Constructed from data of Chubb *et al*. 2008 and Soares *et al*. 2011]

3.2 INTERACTIONS AND SUBCELLULAR LOCALIZATION

DISC1 is predicted to interact with itself forming dimers and oligomers, but also with several other proteins. As no enzymatic activity could be found, DISC1 seems to act as a molecular scaffold, binding more than 200 interactors, which in turn can interact with one another, suggesting an involvement in common pathways (see below)[179–181]. Those binding partners, such as APP, α-tubulin, ATF4/5, dysbindin, FEZ1, Grb2, GSK3β, LIS1, MAP1A, NDEL1, PCM1 or PDE4B/D, are broadly associated with centrosomal and cytoskeletal organization and biogenesis, mRNA/protein synthesis, cell cycle division, intracellular transport and signal transduction processes. Interestingly, DISC1 seems to share binding partners with dysbindin, another risk factor for chronic mental disorders[181].

DISC1 transcripts have been found in brain, heart, placenta, pancreas, testis and kidney[80]. Highest expression levels are found in the dentate gyrus and other regions of hippocampus, cerebral cortex, olfactory bulb and cerebelum[182]. To notice that the hippocampus has been strongly associated with schizophrenia[147]. DISC1 is expressed in neurons and glial cells, and apparently changes its subcellular localization during different phases of neuronal differentiation[183].

DISC1 protein was found on centrosome and cytoskeleton, interacting with MAP1A1, MIPT3, ATF4/5, LIS1 and NDEL1, which participate on axonal and neurite outgrowth[179]. DISC1 have also been found on neurite growth cones and in synaptic structures associated with FEZ1 and Grb2[81,145].

3.3 FUNCTIONS

DISC1 presumed functions are based on its interacting partners, suggesting that if it acts as a scaffold protein it affects the function of different proteins at different locations. DISC1-related psychiatric disorders are likely to arise through the dysregulation of not just one, but several protein interactions at once, affecting neurodevelopmental and signalling pathways. Therefore, DISC1 plays a role on cAMP signaling pathway, dopamine and glutamate signaling, synaptic activity, myelination, neuronal migration, interacting with several proteins related with the cytoskeleton; neuronal proliferation, by interacting with GSK3β, and differentiation, neurite outgrowth, and adult neurogenesis[174,184]. High levels of DISC1 are found in cells in *dentate gyrus* in hippocampus and interneurons on olfactory bulb, where it participates in different steps of adult neurogenesis[151]. Patients suffering from mental illness show reduced olfactory bulb and hippocampal volume, and olfactory and memory dysfunction[115,185,186].

3.4 DISC1 AND VGF

Our group reported that DISC1 knockdown SH-SY5Y cells produce a significant downregulation of VGF, a nerve growth factor responsive gene[187]. DISC1 apparently does not interact directly with VGF, but instead seems to regulate VGF expression through the phosphoinositide 3-kinase (PI3K) /protein kinase B (AKT) /CREB pathway (Figure GI.4)[148]. VGF is a peptide precursor with potent antidepressant effects promoting adult neurogenesis on hippocampus associated with BDNF signalling[150,188–190]. VGF lower-than-normal levels have been associated with schizophrenia, depression, bipolar disorder and other neurological disorders[191–194].

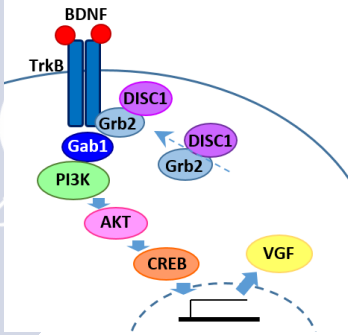

FIGURE GI.4 | PROPOSED DISC1 REGULATION OF VGF.

Trk receptors are activated and recruit Grb2 and Gab2 leading to the activation of PI3K, that activates CREB which in turn promotes VGF expression. DISC1 directly interacts with Grb2 and it is necessary to its proper localization. Lower levels of DISC1 fail to localize Grb2, disrupting this pathway, leading to lower VGF levels.

[Constructed from data of Rodríguez-Seoane *et al*. 2015]

4 NEUROPROTEIN VGF

VGF gene was identified after its strong induction by exposure to nerve growth factor (NGF) in PC12 cells[195]. PC12 is a cell line derived from a pheochromocytoma of the rat adrenal medulla with an embryonic origin from the neural crest that has a mixture of neuroblastic cells and eosinophilic cells, and have been widely used as a model for neural differentiation[196]. VGF was named from the selection of this clone from plate V of Nerve Growth Factor induced PC12 cell cDNA library. *VGF* expression is induced by neurotrophins, as such neurotrophin-3 (NT3) or BDNF through CREB, and by 5-HT and drugs that increase this neurotransmitter levels[189,197,198]. The resulting secreting neuroprotein is widely expressed in neurons in brain, spinal cord and neuroendocrine organs, but regulated in response to different stimuli that originate different smaller peptides, involved in regulation of energy homeostasis, metabolism and synaptic plasticity, and stored in secretory vesicles[199].

4.1 GENE, PROTEIN AND TISSUE DISTRIBUTION

Human *VGF* gene (Ensembl ID: ENSG00000128564) is located at chromosome 7 in the q22 region, and contains 3 exons and several consensus motifs for transcriptional regulators within its promoter. Some of these consensus sequences are a TATA box along with a CCAAT box, for the RNA transcription factor binding, various SP-1 (specificity protein 1) and AP-2 binding sites, an enhancer box, for gene expression regulation through transcription factors, a cAMP response element (CRE) and a CREB binding sites[200–202]. *VGF* expression is induced by activity, such as physical and mental exercise: running, learning, memory, synaptogenesis or long-term potentiation; resulting in an antidepressant-like effect[188,190,203–206]. VGF mRNA is present in neurons of hypothalamus, hippocampus, olfactory system, including olfactory bulb, cerebral cortex, amygdala, brain stem, cerebellum and spinal cord, in motor and sensory neurons and neuroendocrine organs, as pituitary, adrenal gland, and

gastrointestinal tract[207]. VGF mRNA in hypothalamus alters in response to feeding or fasting, salt loading or seasonal rhythms, and in pituitary varies during estrous cycle[208–212].

Full proVGF human protein sequence is encoded only for the 2.3 kb of the third exon originating a 615 residues (617 in mouse/rat) pro-neuroprotein with predicted 68 kDa (Figure GI.5), but detected as a 80-90 kDa doublet. This acidic protein is proline and glycine rich, and belongs to the family of granins, a family of proteins responsible for controlling the endocrine, neuroendocrine and neuronal cells supply of neurotransmitters, hormones, growth factors and peptides[213,214]. ProVGF is a precursor that is processed into more than 15 different neuropeptides (Figure GI.5) by prohormones convertases PC1/3 and PC2 at its C-terminal, but the known N-terminal peptides are not compatible with the known motifs required for PC enzymes, meaning that some VGF-derived peptides are generated by other endoproteases yet not identified[199,215–219]. After proteolytic process in a post-endoplasmic reticulum compartment the derived peptides are kept and enriched in vesicles, and released in response to membrane depolarizing stimuli, playing several roles in neuronal communication [199,220].

4.2 VGF-DERIVED PEPTIDES AND THEIR FUNCTIONS

Studies in mouse, rat, bovine and human revealed several VGF-derived peptides. VGF gene and protein are very highly conserved in these species and one can expect to find the same derived peptides in all those species. Several N-terminal VGF derived peptides were identified in mouse or rat and its correspondents in human: APPG-37, APPG-40, GRPE-37, neuroendocrine regulatory peptides (NERP) 1, 2 and 3, and big NERP-2[194,221]. VGF C-terminal derived peptides were also identified in mouse, rat, bovine and human. Peptide ELQE-20 found in mouse[222] and peptides HFHH-10, HFHH-51 and HHPD-41 were found in rat, the last exhibits bioactivity, but until date no human analogous have been reported[223] . Peptide GGGE was found in rat and its analogous in human GGEE have been described. Peptides TPGH, NERP-3, NERP-4, NAPP-129, TLQP-62, TLQP-21, TLQP-24 (antimicrobial peptide), AQEE-30 (peptide V), AQEE-11, LQEQ-19, were found in mouse, rat and human, and shown to be bioactive.

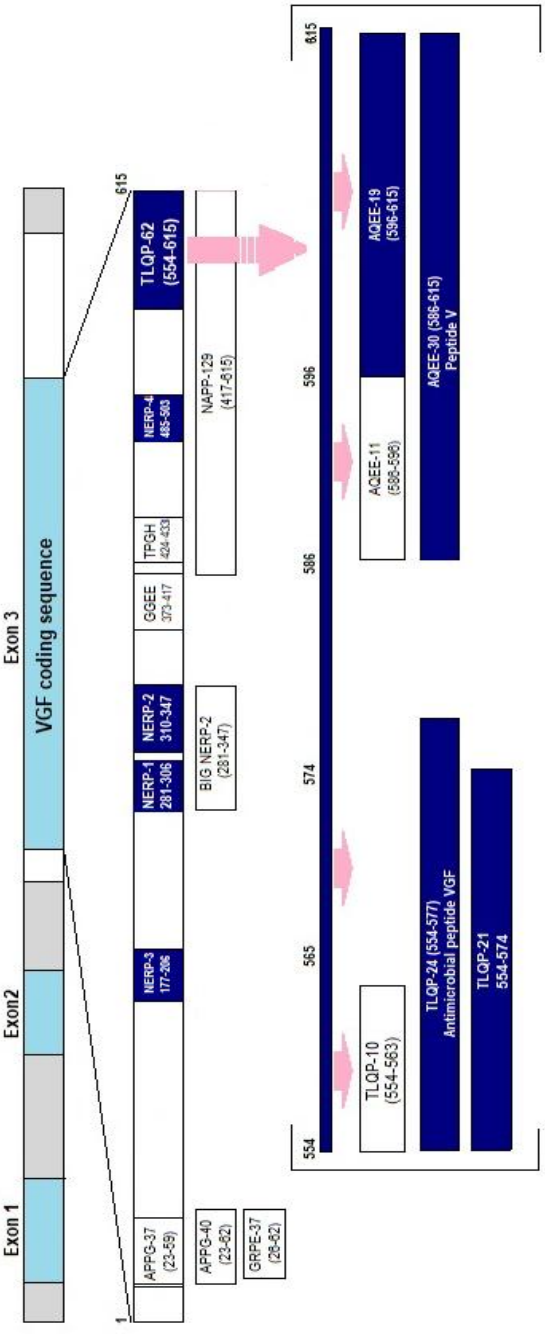

FIGURE GI.5 | SCHEMATIC REPRESENTATION OF HUMAN *VGF* GENE AND ITS DERIVED PEPTIDES. VGF gene encodes a 615 amino acid precursor that is processed in several peptides. In dark blue are the peptides that have been described as showing some biological activity or function. The VGF C-terminal neuropeptide TLQP-62 is **further** processed in several smaller peptides with known biological activities.

TLQP-62 and AQEE-30 have been reported to increase activity of hippocampal neurons, regulate synaptic function, induce neurogenesis and have antidepressive like properties[188,191]. HHPD-41, AQEE-30, AQEE-11 and LQEQ-19 stimulate sympathetic outflow facilitating penile erection in rats[224,225]. NERP-1, -2 and -3 regulate water homeostasis and suppress vasopressin release[226,227]. NERP-2 regulates food intake, gastric acid secretion, body temperature and oxygen consumption, while TLQP-21 increases energy expenditure, prevents diet induced obesity, stimulates catabolic pathways, regulates contractile response in the gastrointestinal tract, has analgesic properties modulating pain, decrease blood pressure and reduces neuronal apoptosis *in vitro*[75,228–234]. Thus, VGF-derived peptides have roles in energy and water balance, gastrointestinal motor function, reproduction, pain, and memory, learning and depression.

VGF-derived peptides are (most of them, but not all) named after its first 4 amino acids and its residue length.

4.2.1. Role in Energy and Water Balance

Fasting has been shown to increase VGF mRNA expression and the administration of leptin prevents this increasing[235]. A $VGF^{-/-}$ mouse model shows no difference at birth from their wildtype or heterozygous counterparts: no defects in development of the central or peripheral nervous systems. However in the following weeks the $VGF^{-/-}$ mice were smaller with less abdominal fat, leaner, more hyperactive and more hypermetabolic than the wildtype mice[208]. Consumed twice as oxygen, and leptin, glucose, insulin and glycogen levels were reduced[236]. Furthermore, VGF deletion blocked obesity in mice in a high-fat diet[235]. VGF seemed to promote an anabolic drive, but further studies showed that intra-cerebroventricular (ICV) infusion of TLQP-21 in mice resulted in a decrease in food intake and a small increase in energy expenditure[228]. Also, mice fed high-fat diet and treated with TLQP21 showed decreased body weight and white adipose tissue, and attenuated rises in leptin, indicating that this peptides blocks the effect of this diet and activates adrenal medulla and adipose tissue. Although VGF seems to have an anabolic role, TLQP-21 shows catabolic activity, which might indicate that different VGF-derived peptides have opposite regulated functions. In fact, further studies showed that administration of TLQP-62, HHPD-41 or

NERP-2 increased food intake, body temperature, oxygen consumption or locomotor activity[209,237,238]. NERP-1 and NERP-2 are expressed in human pancreas islets and inhibit glucose-stimulated insulin secretion levels, by diminishing the number of insulin granules released. Also, insulin granule-related proteins and mRNA are downregulated by NERP-2, and NERP-2 levels are much increased in diabetic pancreas, indicating that NERP peptides might be suppressors of glucose-dependent insulin secretion[239]. TLQP-62 was found to modulate insulin secretion, by increasing basal insulin secretion and glucose-stimulated insulin secretion via increased intracellular calcium mobilization and fast expression of insulin 1 gene. Also, peripheral injection of this peptide improved glucose tolerance in mice, suggesting TLQP-62 is an isulinotropic peptide[240].

Water deprivation and salt loading in rat increases the levels of VGF and vasopressin mRNA and NERP peptides were found to colocalize with vasopressin in storage granules[210,241]. NERP-1, NERP-2 and NERP-3 suppresses the increase in vasopressin induced by hypertonic saline inoculation or water deprivation in rats[227,241]. Thus, NERP peptides may have a role in the control of body fluid balance.

4.2.2. Role in Gastrointestinal Function

ICV injection of TLQP-21 inhibits gastric secretion and emptying by stimulating the release of somatostatin and prostaglandins[242,243]. TLQP-21 induces a gastric contractile motor effect mediated by central nervous system. As stomach plays a primary role in feeding, this inhibition of gastric emptying by TLQP-21 probably represents a signal of satiety and together with this peptide induced increase of energy expenditure[228,229] could be a further mechanism to prevent weight gain and obesity. On the other hand, NERP-2 colocalizes with orexin-A in the lateral hypothalamus and increases orexin-A-induced feeding and energy expenditure. Orexin-A modulates gastric function and ICV administration of NERP-2 increased gastric secretion and emptying through orexin pathway[234].

4.2.3. Roles in Reproduction

VGF gene deletion causes infertility in both male and female mice, with delayed on the onset of puberty and sexual maturation in males and no mature follicles or *corpus lutea*, and reduced ovaries and

uteri in females[208]. Reproductive deficits of VGF$^{-/-}$ seem to arise from deficits in the hypothalamic-pituitary-gonadal axis. As alterations in energy metabolism can affect reproductive function and VFG$^{-/-}$ mice have reduced leptin, it could be suggested that the deficit may be due to gonadotropin releasing hormone (GnRH) synthesis or secretion. However, GnRH levels are not affected but LH and FSH mRNA levels are reduced in VGF$^{-/-}$ mice[208]. Administration of TLQP-21 in female rats during pubertal transition increased the number with signs of ovulation, which might happen through stimulation of GnRH release, as TLQP-21 induces LH secretion[244,245]. Repeated administration of TLQP-21 on adolescent males with chronic food deprivation the gonadotrophin response of hypothalamic-pituitary-gonadal axis was reduced[244]. Other VGF-derived peptides have a role in reproduction. AQEE-11, LQEQ-19, AQEE-30 and HHPD-41 have been shown to induce penile erection in rats in a dose dependent manner and NERP-1 has a pro-erectile effect by acting in the arcuate nucleus, possibly via nitric oxide activation of oxytocinergic pathways[224,246].

4.2.4. Role in Pain Modulation

VGF gene is commonly upregulated in sensory neurons in models of neuropathic and inflammatory pain, and peripheral nerve trauma, colocalizing with substance P, calcitonin gene related peptide and TrkA[230,232,247]. VGF-derived peptides have been associated with pain modulation. Intrathecal infusion of TLQP-62 leads to cold behavioral hypersensitivity in rats, and injection of TLQP-21 in mice hind paw results in hypersensitivity in a formalin model of inflammatory pain[230,248]. TLQP-21 induces thermal hyperalgesia in the warm-water immersion tail-withdrawal test[249]. These analgesic effects and inflammatory modulation depend on the route of administration, being pro-nociceptive at a periphery level and anti-nociceptive at a central level. Hyperalgesia and hypersensitivity induced by TLQP-21 seem to be mediated through activation of macrophages[232]. AQEE-30 and LQEQ-19 have been shown to cause hyperalgesia by inducing mitogen activated protein kinase phosphorylation[247]. Thus, VGF-derived C-terminal peptides seem to play a role in chronic pain in diverse models.

4.2.5. Role in Memory and Learning

VGF mRNA expression has been detected in the hippocampus and to be induced by BDNF exposure, in hippocampal slices, and activities such exercise, memory and learning[188,204]. TLQP-62 has been shown to induce potentiation in hippocampal slices, enhance synaptic activity, increase neurogenesis in adult hippocampus, enhancing dendritic branching and outgrowth, and have an effect in memory[150,190]. Proteomic studies showed a reduction in VGF-derived peptides in cerebrospinal fluids and in parietal cortex of patients affected by Alzheimer's or Parkinson's disease[194]. Moreover, VGF levels have been found to be decreased in depressed patients, but to be restored to normal levels by antidepressant drugs and exercise[192,205]. Microinjection of TLQP-62 into hippocampal CA1 regions showed antidepressant-like behavioral effects in mice via a BDNF-dependent mechanism[250,251]. TLQP-21 exhibits a neuroprotection effect by preventing apoptosis of cerebellar granule cell cultures induced by serum deprivation, and AQEE-30 induces the expression of genes mediating neuronal protection[205,252].

Physical exercise increases VGF expression in mice hippocampus and upregulates a neurotrophic signaling cascade thought to underlie the action of antidepressants.

4.3 ROLE IN NEUROGENESIS AND NEUROPSYCHIATRIC DISORDERS

VGF has been widely explored for its role in emotional behavior and neuropsychiatric illness[213], since its expression pattern is altered in several of those disorders and has even been proposed as a candidate biomarker for frontotemporal dementia[253], amyotrophic lateral sclerosis[254], Parkinson's and Alzheimer's diseases[194], or acute encephalopathy[255]. As described before, VGF has also been found to be involved in depression, bipolar disorder and schizophrenia, being reduced in leukocytes of depressed patients as in the brain of animal models of depression[192], in human bipolar postmortem brain[193], and in hypothalamus and cerebrospinal fluid of schizophrenic patients[256,257]. On the other hand, VGF levels were found to be increased in the hippocampus by antidepressant treatment or voluntary exercise[189,205].

Abnormal activity in the hippocampus has been proposed to play an important role in the origin of depression as evidences suggest that

neurotransmission is disrupted in major depression patients due to changes in synaptic activity[258]. Administration to hippocampal cells of TLQP-62 and AQEE-30 produced an increase in synaptic charge what could explain this peptides antidepressants effects[188]. Chronic TLQP-62 administration induces the BDNF/TrkB/CREB signaling pathway and increases the proliferation of neuronal progenitor cells in the hippocampus in mice, suggesting that this route mediates the anti-depressant effects of this peptide[150,251].

VGF-induced neurogenesis is apparently mediated through NMDA receptor and mGluR5[150]. Activation of mGluR5 induced the phosphorylation of protein kinase D (PKD) in hippocampal neurons and in neural progenitor cells[259]. PKD modulates DNA synthesis and cell proliferation through ERK signal pathway and has anti-apoptosis properties in tumor cells[260]. NMDAR induces the phosphorylation of CaMKII, which regulates synaptic maturation[261,262].

As described, VGF was found to be markedly decreased as a result of DISC-1 silencing in SH-SY5Y neuroblastoma cells, and another study found Neuronal PAS domain protein 3 (NPAS3), a transcription factor associated with risk factor for mental illness, to markedly upregulate and activate VGF through nuclear factor kB (Nf-kB) signaling pathway[263,264]. NPAS3 enhances proliferation of neural cells through VGF, requiring synaptic activity, and PKD and CaMKII molecules through glutamate receptors. Three independent proteins with strong association with neuropsychiatric disorders, DISC1, BDNF and NPAS3, seem to have VGF as a downstream effector, making this neuroprotein, its derived peptides and specially the receptors of the VGF-derived peptides, a very attractive pharmaceutical target to develop new drugs more effective in the treatment of these illnesses. The C-terminal VGF-derived peptides seem to be the ones responsible for this neuroprotective and antidepressant effects, especially TLQP-62 and AQEE-30. Also, given TLQP-21 role in energy homeostasis, it is worth to mention that abnormalities in glucose regulation with increased risk for diabetes mellitus have also been reported in mental disorders, although it is not clear yet if this is related with the antipsychotic drug treatment[265–268].

5 TLQP-62 ANTIDEPRESSANT PEPTIDE

TLQP-62 was first described in 1995 and named after its first four N-terminal amino acids and its peptide length, as happens with several (but not all) other VGF-derived peptides[216]. TLQP-62 is derived from VGF precursor protein via proteolytic cleavage by prohormone convertases PC1/3 at the RPR555 site, and several other VGF-derived peptides are part of this C-terminal neuropeptide, as such TLQP-21, TLQP-24, AQEE-30, AQEE-11, LQEQ-19 (Figure GI.5 and GI.6)[223].

FIGURE GI.6 | VGF-DERIVED TLQP-62 PEPTIDE PRIMARY SEQUENCE. TLQP-62 is formed by the last 62 C-terminal amino acids of proVGF and also integrates peptides TLQP-21 (light blue), AQEEA-30 (underlined), AQEE-11 (dark pink) and LQEQ-19 (purple).

5.1 FUNCTIONS

Among other bioactive VGF derived peptides, TLQP-62 is a very interesting and attractive target for its various behavioural and physiological roles and functions. As described above, TLQP-62, as a bioactive VGF derived peptide plays a role in:

Pain modulation: inducing hypersensitivity to mechanical and cold stimuli, and an increase of VGF mRNA and protein was observed in injured dorsal root ganglion neurons, central terminals and their target dorsal horn neurons[248];

Insulin secretion and glucose homeostasis modulation: TLQP-62 improves glucose tolerance *in vivo*, modulating insulin secretion, by increasing basal insulin secretion and glucose-stimulated insulin secretion via increased intracellular calcium mobilization and fast expression of insulin 1 gene in cultured insulinoma cells. Peripheral injection of this peptide improved glucose tolerance in mice[240].

Memory and learning: TLQP-62 enhances and regulates memory formation mediated by BDNF/TrkB/CREB signalling leading to the expression of genes necessary for memory consolidation, among them BDNF and VGF itself, creating a positive regulatory loop during memory training[190].

Synaptic plasticity: increasing synaptic activity in hippocampal neurons and potentiating CA1 field excitatory postsynaptic potential in hippocampal slices and increasing dendritic branching and length in cultured hippocampal neurons[149,188,250]

Neurogenesis: through this synaptic plasticity, this peptide enhances hippocampal neurogenesis by promoting gene expression and cell proliferation in neuronal progenitor cells.

Antidepressant effect: these effects in synaptic plasticity and neurogenesis are linked to the antidepressant-like effect of this peptide, that when infused in mice hippocampus causes a decrease in forced swim test time reaction and tail suspension test. These effects are mediated through BDNF/TrkB/CREB signaling pathway.

5.2 SIGNALING PATHWAYS

Some studies concerning the elucidation of signaling pathways of TLQP-62 mediated biological functions have been described.

In ***insulin secretion and glucose homeostasis modulation***, TLQP-62 apparently binds an unknown receptor which triggers an intracellular signaling pathway by dephosphorylating AMP-activated protein kinase (AMPK), followed by phosphorylation of ERK and protein kinase C (PKC), increasing the intracellular Ca^{2+} concentration which allows insulin secretion but also TLQP-62, and at the same time induces some genes expression and regulation[240].

Neurogenesis induced by TLQP-62 is mediated through BDNF/TrkB/CREB signaling pathway, and the TrkB activation by BDNF may trigger three main routes: phospholipase Cy (PLCy), PI3K and ERK cascades leading to the phosphorylation of CREB[150,190].

Synaptic plasticity potentiated by TLQP-62 is mediated through BDNF/TrkB activation that can modulate different ion channels (Na+, Ca2+ and K+). BDNF also enhances glutamatergic neurotransmission and the probability of NMAD and mGluR5 receptors activation, further activating CaMKII and phosphoinositide-dependent kinase (PDK), respectively, triggering a cascade modulating DNA synthesis and cell proliferation (Figure GI.7)[150,259].

FIGURE GI.7 | VGF/TLQP-62 PROPOSED REGULATION OF NEUROGENESIS THROUGH BDNF.
The mechanism of action is still unknown, but TLQP-62 enhances he expression of BDNF that interacts with TrkB receptor that are activated and recruit Grb2 and Gab2 leading to the activation of PI3K, that activates CREB which in turn promotes VGF expression and other genes involved in neurogenesis. Also, glutamate receptors NMDA and mGlu5R are activated and required for synaptic activity induced by VGF/BDNF.

Given that TLQP-62 plays a crucial role in the brain, especially in the hippocampus, this neuropeptide is a very attractive target for further search and investigation of its role in neurogenesis, learning, memory and mental disorders, concerning that glucose regulation is also important for maintaining these functions properly.

As no TLQP-62 receptor has been identified so far, the identification of the signalling pathways mediated by TLQP-62 (and its possible connection with DISC1) and of the TLQP-62 receptor(s), as well as both three-dimensional structures, is crucial for better understanding the molecular mechanisms of action of this neuropeptide for further investigation of agonists to be used as a treatment for chronic mental disorders.

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OBJECTIVE

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OBJECTIVE

The global aim of this work was to investigate the molecular mechanisms behind the effect of human VGF-derived antidepressant peptide TLQP-62 on neurogenesis, as this process contributes to memory and learning processes, and is also mechanistically involved in some neuropsychological disorders. For that purpose SH-SY5Y cells were used as an *in vitro* model, as this neuroblastoma cell line can undergo differentiation and adopt a more neuron-like morphology with dopaminergic characteristics.

To achieve this global aim, three specific aims were pursued:

I. Evaluate the effect of TLQP-62 on SH-SY5Y cells, on proliferation and differentiation, as well as its connection with DISC1, by morphological changes and using a proteomic evaluation of TLQP-62 effect on RA-differentiated cells.

II. Identify a receptor for TLQP-62 to better understand how TLQP-62 exerts its effects on neurogenesis, proliferation and differentiation, leading to its known antidepressant effect, and what are the molecular pathways involved, it is of great importance knowing its human receptor(s), which can be latter used as a pharmacological target in treating neuropsychiatric disorders, as depression.

III. Determinate the structure of TLQP-62, once knowing its three-dimensional structure is of great importance when trying to understand how it acts, how it couples with its receptor and how to design future pharmacological drugs to be used as treatment for some neurological impairment.

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CHAPTER I

Effect of human TLQP-62 on SH-SY5Y differentiation

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CHAPTER I

Effect of human TLQP-62 on SH-SY5Y differentiation

1 INTRODUCTION

The VGF-derived TLQP-62 neuropeptide is known for having antidepressant effects and increase memory on the mouse hippocampus by promoting neurogenesis and synaptic plasticity. As this peptide might be implicated in the regulation of neurological functions, it might be crucial to better understand its effects and molecular mechanisms at a neuronal level.

VGF is expressed in neuroendocrine systems and its derived peptide TLQP-62 has been detected at its highest levels in the hippocampus. Several studies have been performed in mice and mouse cultured primary hippocampal neurons showing that BDNF induces VGF expression and in turn VGF increases BDNF expression, enhancing the synaptic activity of those cells, as well as cell proliferation, by increasing the number of type 2a neural progenitor cells by increasing Cyclin D mRNA expression, and dendritic growth and maturation[149,150,188,269]. The activation of BDNF and its receptor TrkB is important for the development of short term memory and neurons growth, and, thus, for neurogenesis.

VGF is induced by several factors, including BDNF, NGF and NPAS3, and in turn VGF is known to induce BDNF expression through BDNF/TrkB/CREB signalling pathway. BDNF is thought to modulate synaptic transmission through mitogen-activated protein kinase kinase (MEK)/ERK and PI3K pathways. NMDA glutamatergic receptor is suspected of being involved in learning and memory by being modulated by BDNF. By inducing BDNF, VGF and its derived peptides TLQP-62 and AQEE-30 are a mechanism to sustain the

functions described above and might act through different routes to exert its effects. However it is not clear how VGF and BDNF promote the expression of each other and which are the specific mechanisms of action and the signaling pathways involved. As schematized in figure I.1, BDNF binds to its receptor, TrkB, which in turn can activate three different pathways: PLCy pathway, implicated in cell adhesion, migration and synaptic plasticity; PI3K pathway, whose phosphorylation leads to the activation of AKT, by PDK, mediating cell survival, growth, proliferation, or differentiation, by phosphorylating several proteins; and ERK pathway, through phosphorylation of Ras, that activates Raf, which in turn activates MEK, which activates ERK, which activates ribosomal protein S6 kinase a1 (S6K1) leading to phosphorylation of transcription factors involved in the activation of genes related too differentiation, maturation and neurite growth[270]. TLQP-62 might participate directly in the binding BDNF/TrkB, as no receptor for this peptide could be identified so far, but could also have not one but several receptors, depending on the tissue and function, which can result in the activation of different signaling pathways.

As previously described, DISC1 is a genetic risk factor implicated in major mental disorders and has a role in neural development and in adult neurogenesis in the dentate gyrus of the hippocampus[146,147,271]. Being a scaffold protein, DISC1 interacts with many other proteins involved in different signalling pathways: GSK3β enhances neural proliferation, through PI3K/AKT/mammalian target of rapamycin (mTOR) pathway, and NDEL1/LIS1 regulates neuronal migration[146,271]. *DISC1* mutations affect not only early neural development but continue to disrupt neuronal development in the hippocampus into adulthood. DISC1 knockdown leads to loss of dendritic branching and neuronal maturity in adult hippocampus causing deficit in short-term plasticity[144,272–274]. Regulation of dendritic development of newborn neurons by DISC1 during adult hippocampal neurogenesis requires neurotransmitter GABA through a convergence onto the AKT-mTOR pathway[143]. Moreover, DISC1 seems to indirectly regulate VGF expression through the PI3K/AKT/CREB pathway[148].

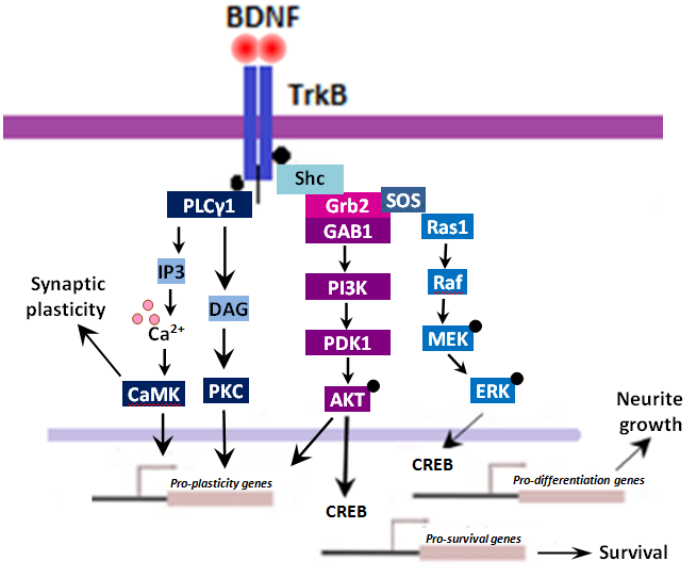

FIGURE I.1 | BDNF/TRKB SIGNALLING PATHWAYS.
[Constructed from data of Castrén *et* Kojima, 2017]

Thus, investigating and understanding more about the roles played by DISC1 and VGF/TLQP-62 on neurogenesis and dendritogenesis could provide new tools and targets to treat neuropsychiatric illnesses.

Culture of primary cells from mouse and rat have been proven useful to study many aspects of neuron physiology and several disease animal models, but human derived cell are necessary to validate the molecular mechanisms of human diseases, which cannot be completely reproduced in animals, and are more adequate for drug testing and screening. The human derived cell line SH-SY5Y reproduces biochemical and morphological properties of neurons, being often used as *in vitro* model for human neurons[275]. This line is a successive sub-clone of the SK-N-SH lineobtained from bone marrow biopsy of a patient with neuroblastoma[276]. SH-SY5Y cells can be induced to differentiate into cells with neuron-like phenotype and expresses high levels of VGF[192,277,278]. VGF is also decreased in SH-SY5Y cells silenced for DISC1, indicating its indirect regulation[187].

The SK-N-SH parental line comprises two morphologically and biochemically distinct phenotypes: neuroblastic (N-type) and substrate adherent (S-type)[277]. The SH-SY5Y line retains a low proportion of S-type cells, but a considerable proportion of N-type cells undergo differentiation into a more neuronal phenotype, with loss of the round morphology and exhibiting neurite extensions, in the presence of *all-trans*-retinoic acid (RA) for 5 days in complete medium (DMEM plus 15% fetal calf serum) and then in the presence of BDNF or NGF[279,280]. Apparently, S-type cells do not exhibit morphological changes and keep proliferating after 10 days of RA-treatment[281]. This effect is caused by the RA-treatment which induces expression of functional TrkB-receptors, which expression is lacking on neuroblastoma cells, making cells responsive to BDNF[282]. BDNF, in turn, activates PI3K, AKT and ERK pathways that mediate survival and neuritogenesis, and so adding BDNF to the RA differentiated culture promotes longer neurites and cells to connect[283–286]. As VGF and TLQP-62 induce BDNF, it would be of interest to evaluate the effect of TLQP-62 on proliferation and differentiation of this cell line, as also investigate the underlying molecular pathways by characterizing the proteomic changes of SH-SY5Y cells after RA/TLQP-62 induced differentiation.

2 OBJECTIVE

The objective of this chapter is to determine the biological effect of TLQP-62 on the human neuroblastoma-derived cell line SH-SY5Y by:

i) evaluating cell morphology, proliferation and differentiation,
ii) comparing those effects with retinoic acid-induced effects,
iii) investigating those effects on VGF and DISC1 silenced cells,
iv) performing a 2D-DIGE proteomic comparative study.

3 METHODS

3.1 HUMAN TLQP-62 PEPTIDE

TLQP-62 (human, molecular weight 7503 Da) and TLQP-21 (human, molecular weight 2491) were purchased from ChinaPeptides Co. LTd., Shangai, with a purity >95%, confirmed by HPLC and MS analysis, as a lyophilized powder. A 1 mg/mL stock solution of each peptide was performed in filtered PBS with 10% acetonitrile and stored at -80ºC. TLQP-21 and TLQP-62 peptide sequences are:

TLQP-62 **TLQPPSALRRRHYHHALPPSR** 21] TLQP-21
HYPGREAQARRAQEEAEAEER 42
RLQEQEELENYIEHVLLRRP 62

3.2 SH-SY5Y CELL CULTURE

SH-SY5Y cell line (European Collection of Cell Cultures, 90430304) is a cloned subline of the neuroblastoma cell line SK-N-SH established in 1970 from a metastatic bone tumor of a four years old female[276,287]. Cells were maintained in a 1:1 proportion Earle's Balanced Salt Solution (EBSS; Sigma-Aldrich) and F12HAM (Sigma-Aldrich) medium, supplemented with 15% Fetal Bovine Serum (FBS; Gibco, Life Technologies), 1% L-glutamine 200 mM (Gibco, Life Technologies), 1% MEM-Non Essential Amino Acids (NEAA; Gibco, Life Technologies) and 1% penicillin-streptomycin (P/S; Gibco, Life Technologies). Cells were seeded at a density of 10^4 cells/cm^2 in 100x20mm culture dishes (Falcon, Life Sciences), previously coated with 0.1 mg/mL collagen (Sigma) and grown at 37ºC in a 5% CO_2 humidified incubator. Media was changed each two days and cells grown until confluence. SH-SY5Y cells silenced for VGF by introduction of shRNA, were previously available in our lab and were used as a negative control and treated the same way as SH-SY5Y cells.

3.2.1. SH-SY5Y preparation for morphology studies

For cellular effect studies of *all-trans*-retinoic acid (RA; Sigma), 10 μM RA, and 1 μM TLQP-62 or 1 μM TLQP-21 were added to the cells the day after seeding in cell medium with 15% FBS. Cells were kept in the presence of RA/TLQP-62/TLQP-21 for 9 days, but switched to 10% FBS medium at day 3 and to 5% FBS medium at day 6. As control SH-SY5Y cells were maintained in 15% FBS cell media for the same period of time. RA was prepared as 1 mg/mL stock solution in 90% ethanol and 10% DMSO. Cells were observed with an Olympus inverted microscope IX51 and the microscope imaging software Olympus CellSens standard. Cells were counted and neurite length determined using ImajeJ software.

3.2.2. SH-SY5Y culture for proteomic studies

For proteomic studies to investigate the effect of TLQP-62 on RA-differentiated SH-SH5Y cells, cells were plated and grown as described before. The day after seeding, 10 μM RA was added to cells in cell medium with 15% FBS. After 3 days, cells were kept in presence of RA but switched to 10% FBS medium and after 3 more days switched to 5% FBS medium and let to differentiate for more 3 days. At day 10 TLQP-62 (1 μM in PBS with 10% acetonitrile) was added to cells for 24 hours for further analysis. As control vehicle solution of PBS with 10% acetonitrile was added to the 9 days RA-differentiated SH-SY5Y cells for 24 h.

3.3 IMMUNOCYTOCHEMISTRY

To confirm the expression and subcellular localization of VGF in SH-SY5Y cells and the knockdown/silencing of VGF on VGFshRNA SH-SY5Y cells, cells were plated and grown as described before in a 4-well Milicell EZ slide (Millipore). At the next day cells were washed twice with PBS and fixed with 10% formalin. For blocking, cells were washed with PBS for 5 min and incubated with 2% BSA in PBS for 30 min. Primary antibodies rabbit anti-human VGF(C-term) was diluted in PBS according to manufacturer instructions and added to the cells for 1 hour at room temperature. Cells were washed 3 times for 5 min with PBS before incubated with the secondary antibody

donkey anti-rabbit IgG-FITC (Santa Cruz Biotechnology) in PBS for 30 min and rinsed with PBS 3 times for 5 min. DAPI was added to the cells (1:1000 in PBS) for 5 min and rinsed with PBS for 5 min, twice. Cells were observed using an Olympus inverted microscope IX51 with an Olympus U-RFL-T reflected fluorescence system and the microscope imaging software Olympus CellSens standard.

3.4 PROTEIN EXTRACTION & QUANTIFICATION

RA-differentiated SH-SY5Y cells incubated for 24 h with TLQP-62 or vehicle solution (control) were washed twice with cold PBS and solubilized in lysis buffer (20 mM HEPES pH 7.4, 2 mM EGTA, 1 mM DTT, 1 mM sodium orthovanadate, 1% Triton X-100, 10% glycerol, 2 µM leupeptin, 400 µM PMSF, 50 µM β-glycerophosphate and 100 µg/ml aprotinin). The cells were scrapped on ice for ten minutes and incubated on ice for 30 min with periodic vortexing at each 10 minutes. Sonication on ice was performed for 3 periods of 15 seconds with 10 seconds interval on ice in between each pulse, with a 10% amplitude. Centrifugation at 4ºC, 14000x*g*, for 30 min was performed. Pellet was discarded and supernatant recovered for further precipitation with 60% trichloroacetic acid (TCA) in acetone. Frozen samples were mixed with half of their volume with 60% TCA/acetone and incubated on ice for 45 min. Samples were centrifuged for 2 min at 10000x*g*, 4ºC, and supernatant was discarded. Pellet was resuspended in 500 µl of cold acetone and sonicated 3-4 pulses in the ultrasonic cell disruptor Sonifier 150 (Branson). Samples were kept on ice and further centrifuged at 10000x*g* for 1 min at 4ºC. Supernatant was discarded and pellet was washed twice with 500 µl of cold acetone. At last wash supernatant was discarded and pellet was left to air dry. Dry pellet was ressuspended in a minimum volume of 2D-DIGE sample buffer (5 M urea, 2 M thiourea, 2 mM tributyl-phosphine, 65 mM DTT, 65 mM CHAPS, 150 mM non-detergent sulfobetaine (NDSB-256), 1 mM sodium vanadate, 0.1 mM sodium fluoride and 1 mM bensamidine), vigorously vortexed for better solubilization and centrifuged for 2 min at 10000x*g*. Supernatant was recovered and quantified.

Protein was quantified using Coomassie Plus Protein Reagent (Thermo Scientific) compatible with the 2D-DIGE buffer used to solubilize the samples. A six different points standard calibration curve was performed in duplicated by serial dilutions of 1 mg/ml BSA in 2D buffer, for six concentrations of BSA: 1000, 500, 250, 125, 62.5 and 31.25 µg/ml. For protein quantification, 20 µl of each curve point, 20 µl 2D buffer (blank) and 2 µl of each sample were added to 600 µl of Coomassie reagent in duplicate. The mixture was let stand at room temperature for 5 min and absorbance was measured at 595 nm in a Genesis 20 Spectrophotometer (thermos Scientific).

3.5 MONODIMENSIONAL (1D) SDS-PAGE

Protein extracts (20 µg) were mixed with Laemmli sample buffer (Bio-rad) with 5% β-mercaptoethanol and boiled for 5 minutes at 99°C and loaded on precast NuPAGE® 4-12% Bis/Tris gels (Invitrogen). SDS-PAGE Molecular Weight Standards, Broad Range (Bio-rad) or Pierce Unstained Protein Molecular Weight Marker (Thermo Scientific). Samples were applied on gel and subjected to electrophoresis in XCell Sure Lock® system (Invitrogen) with electrophoresis buffer MES (Invitrogen). The electrophoresis was performed for 40 min at 200 V and gels were transferred to PVDF membrane for further immunoblotting.

3.6 IMMUNOBLOTTING

Proteins in 1D gels were transferred to Immobilun® Transfer Membrane PVDF (Millipore) after methanol activation. Transfer was performed in a Trans-Blot® Semi Dry Electrophoretic Transfer Cell (Biorad) using Extra Thick Paper® Filter paper (Bio-rad) with transfer buffer (25mM Tris base, 192 mM glycine, 3.5 mM SDS, pH 8.3 and 20% methanol) at 15 V and 0.8 mA/cm^2 for 45 min. PVDF membranes were blocked in 5% BSA (Sigma-Aldrich) in TBS-0.1% Tween-20 (Sigma-Aldrich) (TBS-T) solution overnight at 4°C. Membranes were further incubated 1 hour at room temperature with primary antibody goat anti-human VGF (C-terminal) (Santa Cruz) diluted 1:500 in 1% BSA in TBS-T. Membranes were washed three times for 10 minutes each with TBS-0.1% Tween-20 and further

incubated with donkey anti-goat IgG-HRP (Santa Cruz) diluted 1:2000 in 1% BSA in TBS-0.1% Tween-20 for 1 hour. Membranes were washed in TBS-0.1% Tween-20 for three times for 10 minutes and one last time in TBS for 5 minutes before incubation with chemiluminescent substrate Luminata Forte Western HRP substrate (Merck Millipore). For developing of the membrane, a BioMax Cassette (Kodak), Amersham Hyperfilm ECL (GE Healthcare) and G140 developer and G354 fixing solutions (Agfa) were used in a dark room with red light. The exposure was performed at different times between 1 min to 2 hours.

3.7 BIDIMENSIONAL FLUORESCENCE DIFFERENTIAL GEL ELECTROPHORESIS (2D-DIGE)

This experiment was performed in collaboration with Angel Garcia's Proteomic group from CiMUS, USC. For the final comparative proteomic study of RA-differentiated SH-SY5Y cells, 6 replicas of each sample (control and TLQP-62 induced cells for 24h) were analyzed by 2D-DIGE in large 25x20 cm 10% polyacrylamide gels after labelling with fluorescent dyes.

3.7.1. Fluorescent labelling and Rehydration

Samples were labeled with CyDye DIGE fluor minimal dye labelling kit (GE Healthcare) composed by the cyanine dyes Cy2, Cy3, and Cy5. The fluorescent dyes were reconstituted on anhydrous dimethylformamide (DMF) as stock solutions of 1 nmol/µl. Labelling was performed by adding 0.4 nmol Cy3 or Cy5 to 50 µg of control sample or peptide sample and 0.4 nmol Cy2 to 50 µg of an intern standard constituted by a mix of equal parts of control sample and peptide sample. Samples were left for 30 min in ice and 1 µl lysine 10 mM was added to each sample to stop the reaction 10 min on ice. The 3 differently labelled samples were pooled together and the 1 volume of 2D-DIGE buffer was added and let on ice for 10 min. Samples were mixed with 1.6% (v/v) of ampholytes (pH 4-7; Serva Electrophoresis GmH) and 2D buffer was added to a final 500 µl volume, briefly vortexed and centrifuged to eliminate impurities before rehydration of IPG strips. A total of 150 µg protein was applied to each of the six strips/gels. Table I.1 illustrates constitution of each sample per gel.

After centrifugation samples were pipetted in a lane of the Immobiline DryStrip Reswelling tray (GE, Healthcare) in contact with the gel fragment on the 24 cm Immobiline 4-7 pH gradient IPG strip (GE Healthcare). Strips were covered with mineral oil Dry Strip Cover Fluid (GE Healthcare) to avoid evaporation of the samples and left in 500 µl rehydration buffer overnight. The following day, the strips were placed in a Multiphor II electrophoresis unit (GE Healthcare) with the gel face up to run the first dimension separation.

TABLE I.1 | FLUORESCENT LABELLING OF SAMPLES.

Gel	Cy2	Cy3	Cy5
1	25 µg control sample + 25 µg peptide sample	50 µg control sample	50 µg peptide sample
2	25 µg control sample + 25 µg peptide sample	50 µg peptide sample	50 µg control sample
3	25 µg control sample + 25 µg peptide sample	50 µg control sample	50 µg peptide sample
4	25 µg control sample + 25 µg peptide sample	50 µg peptide sample	50 µg control sample
5	25 µg control sample + 25 µg peptide sample	50 µg control sample	50 µg peptide sample
6	25 µg control sample + 25 µg peptide sample	50 µg peptide sample	50 µg control sample

3.7.2. First dimension: IEF and Equilibration

The IPG strips were covered with mineral oils and the electrodes were set in place for the first dimension to be carried out at 17ºC. Isoelectrofocusing (IEF) conditions are described in table I.2. After IEF, proteins in the strips were equilibrated, reduced and alkylated. Strips were incubated for 15 min at room temperature with agitation in equilibration buffer (2D-DIGE buffer supplemented with 2% DTT), washed with milliQ water, incubated for 15 min at room temperature with agitation in alkylating buffer (equilibration buffer with 2.5% iodoacetamide), and washed again with H_2O milliQ.

TABLE I.2 | ISOELECTROFOCUSING CONDITIONS FOR 24 CM STRIPS.

Phase	Voltage (V)	Amperage (mA)	Wattage (W)	Time
I	150	3	5	2 h
II	300	10	5	2 h
III	1000	10	5	2 h
IV	4000	10	5	16 h

3.7.3. Second dimension: SDS-PAGE

Large 10% polyacrylamide gels of 25x20 cm were prepared the previous day, as described in table I.3, and let to polymerize for 5h at room temperature on a DALT six Gel Caster System (GE Heathcare). Equilibrated strips were immersed in agarose solution 0.5% placed above the polyacrylamide gels avoiding bubbles between the strip and the gel. Electrophoresis was carried out in an EttanDalt six System apparatus (GE Healthcare) with electrophoresis buffer (0.25 M Tris Base, 1.92 M glycine, 1% SDS) at 10ºC for 1 hour at 80 V, 20 mA/gel, 2 W/gel and then 6 h at 500 V, 40 mA/gel and 17 W/gel.

After, each gel was scanned 3 times, each for each dye, in a Typhoon 9410 scanner (GE Healthcare) and Trypoon Scanner Control 5.0 program with 200 µm resolution. For Cy2 with blue laser 488 nm excitation wavelength and 520 band pass (bp) 40 filter; Cy3 with a green laser 532 nm and 580 bp 30; and Cy5 with red laser 633 nm and 670 bp 30. For all with medium sensitivity and photomultiplier 600V.

TABLE I.3 | VOLUME OF REAGENTS USED TO PREPARE THE POLYACRYLAMIDE GELS

Reagents	10% gels (6)
Bis-Acrylamide 30%	150 mL
1.5 M Tris pH 8.8	113 mL
SDS 10%	4.5 mL
H_2O MilliQ	147 mL
APS 10%	4.5 mL
TEMED 10%	0.8 mL
Rinohide	30 mL

Images were taken and the image analysis program Progenesis SameSpots 4.5 (Nonlinear Dynamics Ltd, Newcastle, UK) was used to find real differences between the two study conditions. A spot pattern was created by the program for all six gels and a statistic ANOVA analysis was performed. All spots with a variation ≥1.2 and a $p<0.05$ were considered.

3.8 SYPRO® RUBY PROTEIN GEL STAINING

For protein detection SYPRO® Ruby (Invitrogen) was used according to manufacturer instructions. After SDS-PAGE, gels were washed in H_2O_{MQ} for 10 min, followed by washing for 1 h in 50% methanol and 7% acetic acid solution. Gels were then stained by SYPRO® Ruby solution overnight protected from light, fixed with 10% methanol and 7% acetic acid solution for 1 hour and washed with H_2O_{MQ} for 10 minutes. Gels were visualized in a Gel®Doc system (Bio-rad) and kept in water from light at 4ºC until band excision.

3.9 MASS SPECTROMETRY

The spots chosen for analysis, after SYPRO Ruby gel staining and visualization, were excised from a gel using a Blue Box (Invitrogen) to aid visualization and sent for Mascote protein identification by nLC-ESI-trampa with tryptic digestion at Mass Spectrometry Unit at the *CACTUS* core facilities at University of Santiago de Compostela:

> Excised gel bands or spots were washed three times with 100 µL of 50 mM ammonium bicarbonate in 50% methanol, reduced with 10 mM DTT, washed three times with 50 mM ammonium bicarbonate, let to air-dry and alkylated with 55 mM iodoacetamide. Gel bands were washed with ammonium bicarbonate, dehydrated with acetonitrile and let to air-dry. Porcine modified trypsin (Promega) was added to a final concentration of 20 ng/mL in 20 mM ammonium bicarbonate, and digestion let to occur overnight at 37 ºC. Peptide extraction from gel was carried out with 40 µL 60% acetonitrile in 0.5% formic acid. Extracts were pooled, dried and kept at -20 ºC. Peptide identification was performed using a model Amazon ETD mass Spectrometry spectrometer (Bruker). Resulting MS spectra was analysed for database search with the Mascot v2.1 search tool (Matrix Science, London, UK) screening SwissProt. Searching was restricted to human taxonomy.

4 RESULTS

4.1 CONFIRMATION OF VGF EXPRESSION IN SH-SY5Y CELLS

As TLQP-62 is known for promoting the expression of BDNF[150,190], the effect of this peptide on RA differentiated cells was investigated to evaluate if it is similar to the one observed with BDNF. VGF, specifically TLQP-62, has been described as promoting cell proliferation rather than differentiation[150].

To investigate that effect, an evaluation of the effect of TLQP-62 on SH-SY5Y and RA differentiated-SH-SY5Y cells was performed. To confirm the expression of VGF in these cells, an immunoblotting and immunocytochemistry experiment was performed on SH-SY5Y cells using a rabbit IgG anti-human VGF(C-term) antibody, making possible to confirm VGF expression on those cells and that is mainly kept in vesicles at the cell neurites, as previously described (Figure I.2 and I.3). The same cell line silenced for VGF was used as a negative control and to investigate the role of VGF and TLQP-62 on these cells. In the VGF-silenced cells, almost no signal corresponding to VGF(C-term) can be detected and this decreasing in protein expression seems to lead to a different cell morphology, with a rounder body shape and shorter dendrites, illustrating a role for VGF on dendritegenesis, known to be regulated by PI3K/AKT/mTOR pathway[288].

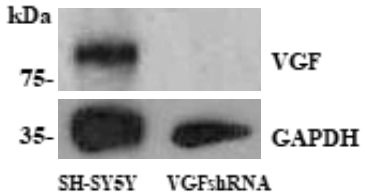

FIGURE I.2 | SH-SY5Y AND VGFSHRNA SH-SY5Y VGF EXPRESSION EVALUATION BY IMMUNOBLOTTING.

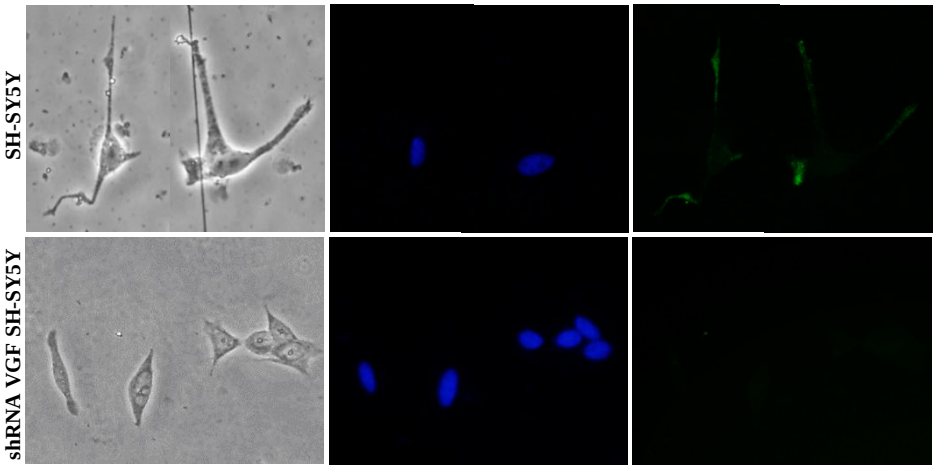

FIGURE I.3 | SUBCELLULAR LOCALIZATION OF VGF (C-TERM) ON SH-SY5Y AND VGF-SILENCED SH-SY5Y CELLS BY IMMUNOCYTOCHEMISTRY.

4.2 TLQP-62 PROMOTES DIFFERENTIATION AND NEURITOGENESIS ON SH-SY5Y CELLS RATHER THAN PROLIFERATION

SH-SY5Y cells were plate grown for 2 days before 1 μM TLQP-62 was added to the cells in order to evaluate its effects on the cell morphology. As previously described, the SH-SY5Y cell line has two morphologically and biochemically distinct phenotypes, N-type and lower proportion of S-type[277]. When cultures are treated with 10 μM RA for 5 days in complete medium (DMEM plus 15% fetal calf serum), a considerable proportion of N-type cells differentiated to a more neuronal phenotype with loss of the round morphology and exhibiting neurite extension[279]. This neuritogenesis enhancement occurs through induction of TrkB receptors and BDNF by RA and PI3K/AKT and ERK/MAPK pathways. As described before, TLQP-62 promotes the expression of BDNF leading to cell proliferation rather than differentiation[150]. Here, an evaluation of the effect of TLQP-62 on SH-SY5Y and RA differentiated-SH-SY5Y cells was performed.

SH-SY5Y cells were plated ($3x10^6$ cells) and after 2 days growth was added to the cultured cells *i)* vehicle control (10% acetonitrile in PBS), *ii)* RA (10 μM), *iii)* TLQP-62 (1 μM) or *iv)* TLQP-21 (1 μM). Cells were let to grow for 3 days and observed for proliferation and differentiation evaluation (Figure I.4). TLQP-62 alone has the capacity of induce proliferation and differentiation. TLQP-21, another VGF-derived neuropeptide implicated on pain and glucose homeostasis, was also investigated for its capacity to promote these effects, or if those are specific of TLQP-62 neuropeptide. VGF-silenced SH-SY5Y cells (VGFshRNA SH-SY5Y) were also used (Figure I.4).

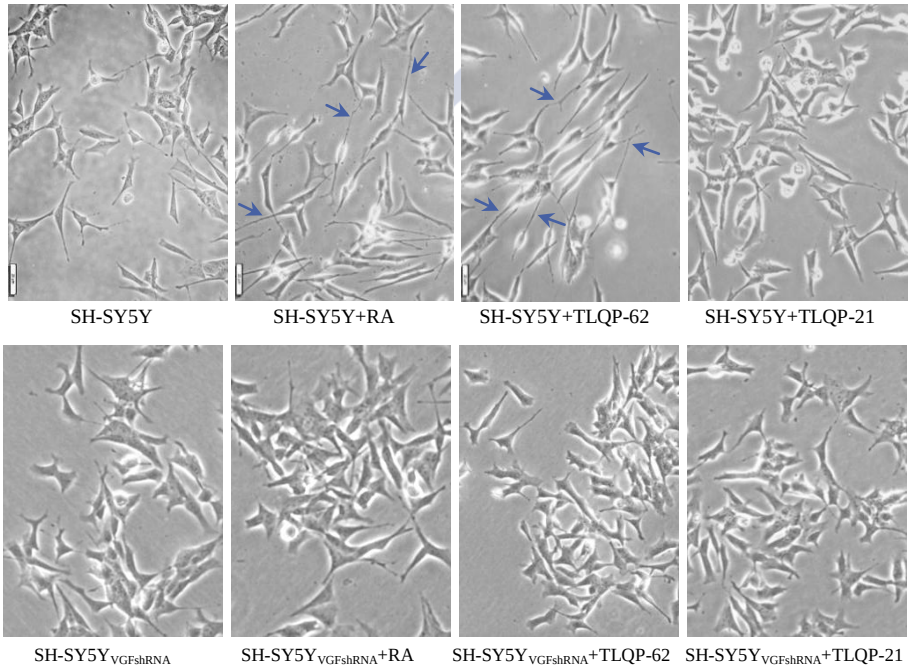

FIGURE I.4 | EFFECT OF RETINOIC ACID, TLQP-62 AND TLQP-21 ON SH-SY5Y AND VGF-SILENCED SH-SY5Y CELL PROLIFERATION, DIFFERENTIATION AND MORPHOLOGY. SH-SY5Y and VGF silenced SH-SY5Y cells were plated and grown for **3 days** and compared to the ones grown for the same time in the presence of 10 μM RA, 1 μM TLQP-62 or 1 μM TLQP-21 in order to evaluate its effects on the cell morphology, differentiation and proliferation. Cells were observed with an Olympus inverted microscope IX51 and the microscope imaging software Olympus CellSens standard. Blue arrows indicate neurite outgrow.

SH-SY5Y cultures treated with 10 μM RA show a considerable proportion of cells differentiated into a more neuronal phenotype with loss of the neuroblastic round morphology and exhibiting neurite extensions that occasionally connect the cells. TLQP-62 apparently has the same effect as RA, inducing some signs of neurite outgrowth level. Besides, after 3 days in the absence of RA or TLQP-62 no changes indicating cell differentiation could be observed, indicating that TLQP-62 might be involved in the RA differentiation pathway and its expression crucial for neuritogenesis.

To investigate the prolonged effects of TLQP-62, the peptide was added to the cultured SH-SY5Y for up to 9 days to evaluate the morphological changes (Figure I.5). TLQP-62 apparently inhibits cell proliferation but induces and sustains a considerable proportion of neuroblastic cells to differentiate into a more neuronal phenotype, with loss of the round morphology and exhibiting neurite extensions that occasionally connect the cells, when compared to the cells not treated for the same period, as also observed in figure I.5.

4.3 TLQP-21 MODERATELY INHIBITS PROLIFERATION OF SH-SY5Y CELLS BUT HAS NO EFFECT ON DIFFERENTIATION

As stated before, TLQP-21, a VGF-derived neuropeptide implicated on pain and glucose homeostasis, and described as preventing cell death, was investigated for its capacity to promote cellular proliferation and/or differentiation on SH-SY5Y cells. Cells were treated for three days with 1 μM TLQP-21 and then evaluated for morphological changes. The cells in presence of TLQP-21 showed less morphological changes when compared to the ones treated with TLQP-62 or RA (Figure I.4). VGF-silenced SH-SY5Y cells show a rounder morphology with shorter neurites when compared with SH-SY5Y cells. Prolonged treatment of SH-SY5Y cells with TLQP-21 for 9 days, showed a moderate inhibitory effect of TLQP-21 on cell proliferation and no effect on cell differentiation, when compared to TLQP-62 effect on the same cells (Figure I.5). TLQP-21 effect was also investigated and same experiment was performed on VGF-silenced SH-SY5Y cells (Figures I.6). No effect was observed on those cells.

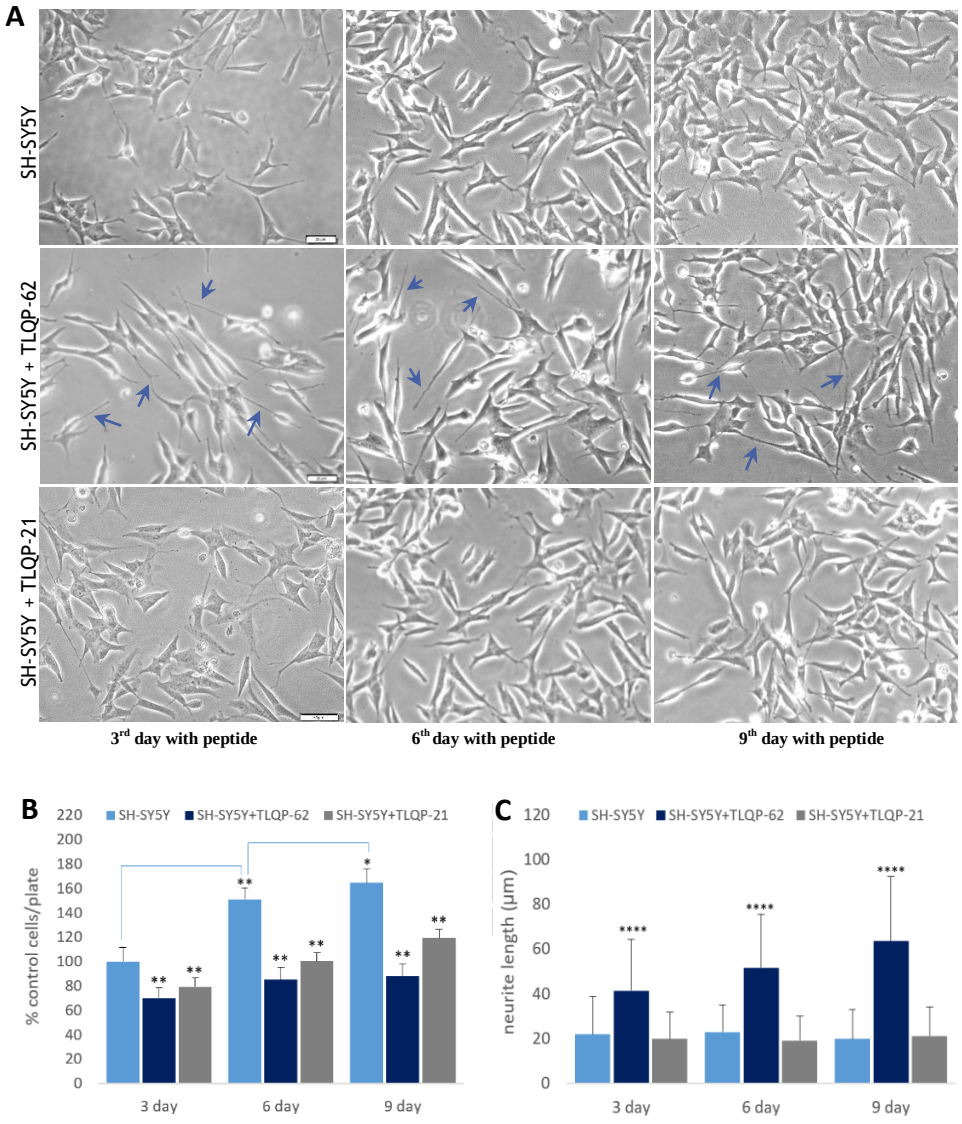

FIGURE I.5 | EFFECT OF TLQP-62 AND TLQP-21 ON SH-SY5Y CELL GROWTH AND MORPHOLOGY.

SH-SY5Y cells were plated and grown for up to 9 days and compared to the ones grown for the same time in the presence of 1 µM TLQP-62 or 1 µM TLQP-21. **A)** Cells were observed with an Olympus inverted microscope IX51 and the microscope imaging software Olympus CellSens standard at 3rd, 6th and 9th days. Cells were evaluated for **B)** average cell growth +/-SD; *p<0.01 and **p<0.005 SH-SY5Y vs treated (Mann-Whitney U test); and **C)** average of neurite length +/-SD; ****p<0.0001, n>100, SH-SY5Y vs treated. Blue arrows indicate neurite outgrowth.

4.4 VGF SILENCING AFFECTS SH-SY5Y CELL VIABILITY

VGF silenced SH-SY5Y cells seem to have a shorter life time when compared to the wildtype ones and to have a lower proliferative rate. After six days on culture, with changing media each 2 days, cells grew at a lower rate when compared to wildtype cells. After nine days in culture some of the surviving cells are smaller and thinner, which gives the idea of some neurite growth (Figure I.6). Apparently, neither TLQP-62 nor TLQP-21 affect positively SH-SY5Y cell differentiation or proliferation, or protect SH-SY5Y from cell death. This might indicate that a higher concentration of TLQP-62 is needed to support TLQP-62 effects on those silenced cells or that VGF and other VGF-derived peptides are required for normal cell survival and proliferation.

4.5 TLQP-62 SUPPORTS SH-SY5Y RA-INDUCED DIFFERENTIATION

To investigate the effects of TLQP-62 in sustaining RA-differentiation, the SH-SY5Y cells were treated for 9 days with RA and then TLQP-62 was added for more 3 days in reduced FBS containing medium (from 15% to 1% FBS), for morphological evaluation (Figure I.7). As control, RA-differentiated SH-SY5Y cells were treated with vehicle control instead of TLQP-62; and the TLQP-21 effect was also investigated. After RA-induced neurodifferentiation in low FBS medium, if RA is taken from the media cells start dying and detach from plate (data not shown). TLQP-62 not only induces differentiation (although at a slower rate when compared to RA) but it also successfully supports SH-SY5Y RA-induced differentiation and survival in low FBS containing medium. Cells not only survive as they keep growing neurites, showing bigger length, and occasionally interacting with each other. Surprisingly, although TLQP-21 alone is apparently not capable of induce SH-SY5Y differentiation, it seemed to be capable to support RA-differentiated cells survival in low FBS, after the absence of RA. Same experiment was also performed on VGF-silenced SH-SY5Y cells as control (data no shown) and the ability to support RA differentiation by TLQP-62 and TLQP-21 was also observed, although there are less differentiated cells, indicating that VGF is crucial for this process.

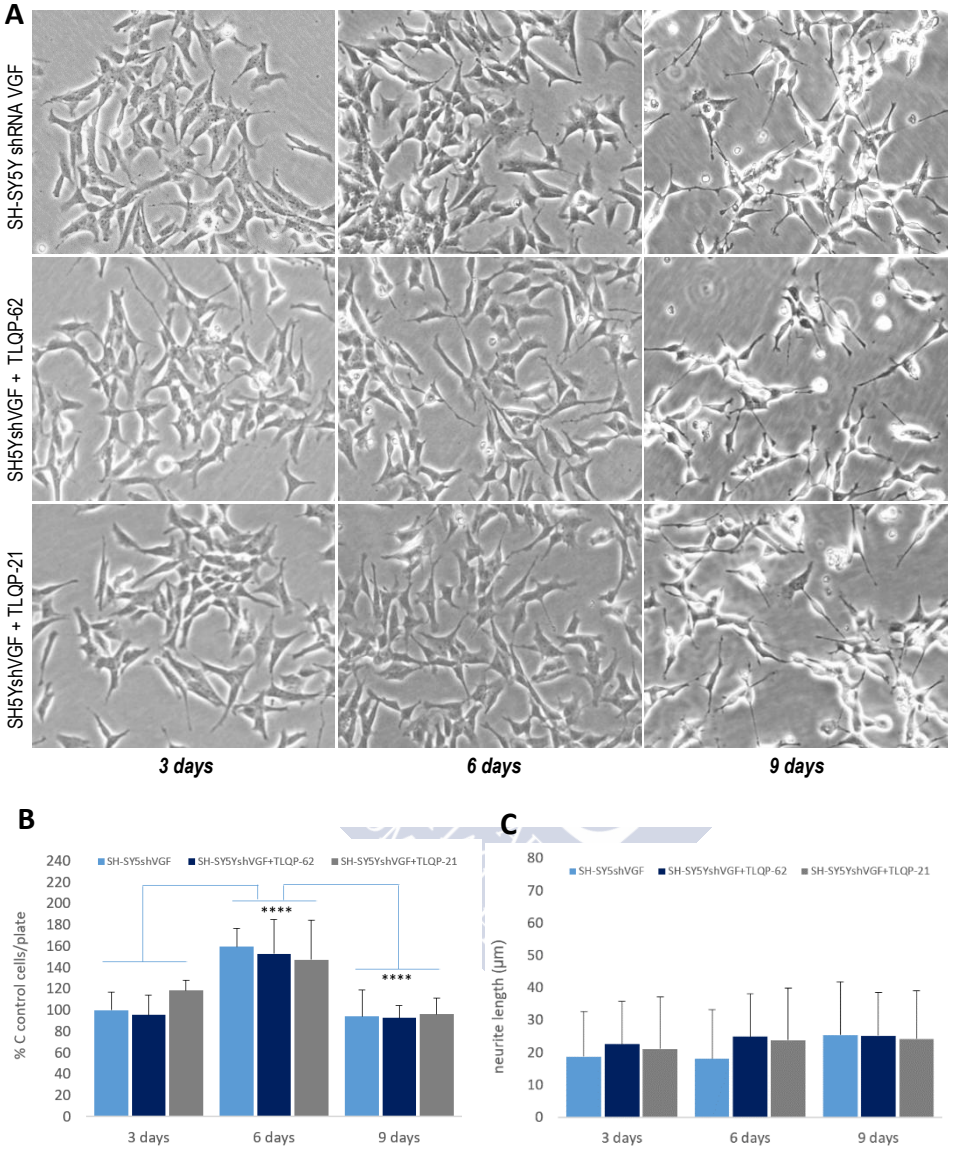

FIGURE I.6 | EFFECT OF TLQP-62 AND TLQP-21 ON VGF SILENCED SH-SY5Y CELL GROWTH AND MORPHOLOGY. SH-SY5Y cells knockdown for VGF were plated and grown for up to 9 days and compared to the ones grown for the same time in the presence of 1 µM TLQP-62 or 1 µM TLQP-21. A) Cells were observed with an Olympus inverted microscope IX51 and the microscope imaging software Olympus CellSens standard at 3rd, 6th and 9th day, and evaluated for B) average of cell growth +/-SD; ****p< 0.0001 (Mann-Whitney U test); and C) average of neurite length +/-SD.

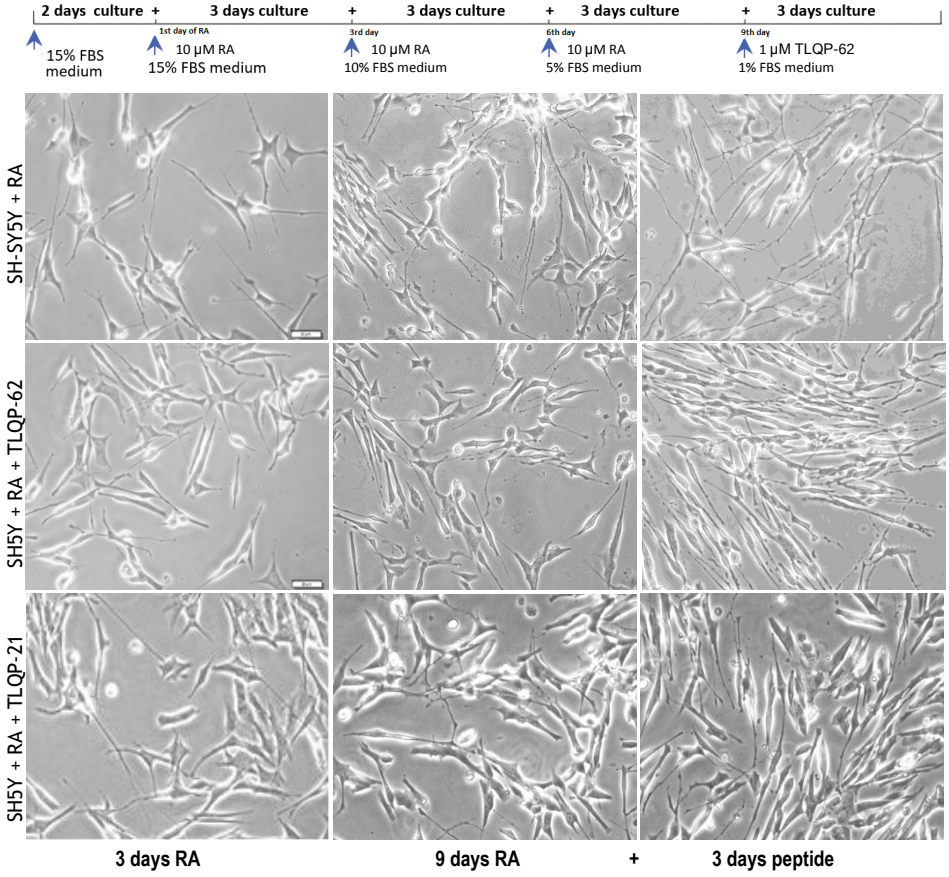

FIGURE I.7 | EFFECT OF TLQP-62 AND TLQP-21 ON RETINOIC ACID-DIFFERENTIATED SH-SY5Y CELL MORPHOLOGY. SH-SY5Y cells were plated and grown for up to 9 days on the presence of 10 µM all-trans RA and compared to the ones grown for 6 days in the presence of 10 µM RA and three more days with 1 µM TLQP-62 or 1 µM TLQP-21, in order to evaluate its effects on the cell morphology, differentiation and proliferation. Cells were observed with an Olympus inverted microscope IX51 and the microscope imaging software Olympus CellSens standard.

4.6 SH-SY5Y MORPHOLOGICAL EFFECTS OF TLQP-62 AND DISC1

As described, DISC1 plays an important role in regulating neurodevelopment, being associated with chronic mental disorders. Moreover, it is known to be involved in neurogenesis and to upregulate VGF expression, apparently through PKA/AKT/CREB pathway.

DISC1 effect on TLQP-62 treated SH-SY5Y cells was investigated. For that purpose, SH-SY5Y cells shRNA-silenced and overexpressing DISC1, under Tet-inducible system control, were used. As positive and negative control, SH-SY5Y cells and shRNA-VGF silenced SH-SY5Y cells, respectively, were used. Those cells were previously available in our laboratory from previous studies.

SH-SY5Y cells were treated with RA for 9 days for morphological evaluation, followed by TLQP-62 treatment for more 3 days. As observed in figure I.12, and previously described in this chapter, TLQP-62 supports RA-SH-SY5Y neurodifferentiation effects, with neuroblastoma cells presenting a more neuron-like phenotype, with more and longer dendrites. As negative control, shVGF-SH-SY5Y cells were used, and, as described, those cells are rounder, with a lower growth rate, and, although RA is capable of induce these cells differentiation, dendrites are fewer and shorter, comparing to the wildtype cells with same treatment. Thus, as already stated, indicates VGF is important for proper cell morphology and differentiation and that TLQP-62 has a role on neurogenesis or dendritogenesis.

In an attempt to understand DISC1 and TLQP-62 connection regarding SH-SY5Y neurodifferentiation, cells silenced for DISC1 were evaluated for their capacity to support RA-induced differentiation. Apparently those cells are morphologically normal, but in the presence of RA, comparing to the wildtype, cells have fewer and shorter dendrites (Figure I.8). After 3 days with TLQP-62 treatment, cells keep exhibiting differentiation with neurite outgrowth. DISC1 overexpression enhances SH-SY5Y neurodifferentiation in the presence of RA, and cells presented longer dendrites when compared to the wildtype ones. Plus, TLQP-62 is, as expected, capable of supporting this induced differentiation.

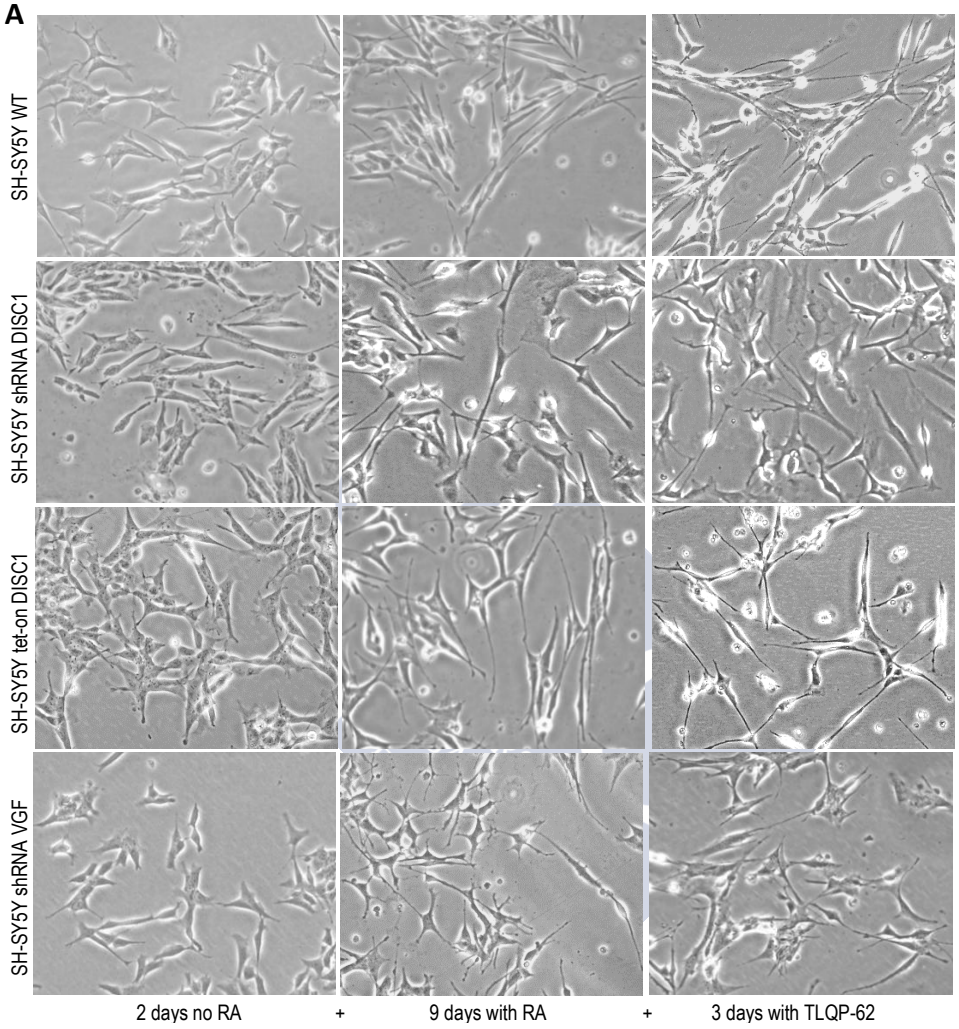

Figure I.8 | EFFECT OF TLQP-62 ON RETINOIC ACID DIFFERENTIATED SH-SY5Y, VGF SILENCED SH-SY5Y, DISC1 SILENCED SH-SY5Y AND DISC1 OVEREXPRESSING SH-SY5Y CELL MORPHOLOGY.
Cells were plated and grown for 2 days and then 9 days on the presence of 10 μM all-trans RA and more 3 days with1μM TLQP-62 in low FBS media in order to evaluate the capability of the peptide to support cell growth and survival, and its effects on the cell morphology, differentiation and proliferation. A) Cells were observed with an Olympus inverted microscope IX51 and the microscope imaging software Olympus CellSens standard, and evaluated for B) average of cell growth +/-SD and C) average of neurite length +/-SD.

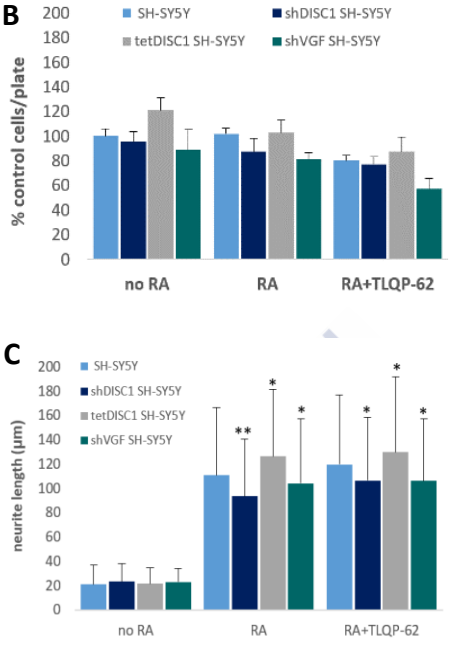

Figure I.8| EFFECT OF TLQP-62 ON RETINOIC ACID DIFFERENTIATED SH-SY5Y, VGF SILENCED SH-SY5Y, DISC1 SILENCED SH-SY5Y AND DISC1 OVEREXPRESSING SH-SY5Y CELL MORPHOLOGY (CONTINUED).

Cells were plated and grown for 3 days and then 6 days on the presence of 10 µM all-trans RA and more 3 days with1µM TLQP-62 in low FBS media in order to evaluate the capability of the peptide to support cell growth and survival, and its effects on the cell morphology, differentiation and proliferation. **A)** Cells were observed at 12th day with an Olympus inverted microscope IX51 and the microscope imaging software Olympus CellSens standard, and evaluated for **B)** average of cell growth +/-SD; and **C)** average of neurite length +/-SD; *p<0.05 and **p<0.01, n>100 (Mann-Whitney U test).

4.7 PROTEOMIC STUDY OF RA-DIFFFERENTIATED SH-SY5Y CELL RESPONSE TO TLQP-62 BY 2D-DIGE: UPREGULATION OF PROTEINS INVOLVED IN NEURODEVELOPMENTAL AND SYNAPTIC PROCESSES, ENERGY METABOLISM, OXIDATIVE STRESS AND IMMUNE RESPONSE

As TLQP-62 seems to support SH-SY5Y RA-differentiation, those SY5Y cells were used to evaluate TLQP-62 proteomic response, once this model better reproduces morphological and biochemical properties of neurons, as cells exhibit a more neuron-like phenotype.

RA-differentiated SH-SY5Y cells have been reported as undergoing apoptosis after 24 h in RA and serum-free medium[281]. Thus, a 2D fluorescence differential gel electrophoresis was performed as a comparative analysis to evaluate in detail the response on the proteasome of RA-differentiated SH-SY5Y cell cultures exposed to TLQP-62 for 24h.

Therefore, differential analysis was performed to total protein lysate from RA-differentiated SH-SY5Y cell cultures incubated with TLQP-62 for 24 h together with the one resulting from cells incubated for the same time with vehicle control, PBS+10%ACN. Six gels containing the analysed samples were analysed and a set of spots was obtained. Among those spots, 39 were found to be differentially regulated, by exhibiting different intensities in both analysed conditions, with a fold change ≥ 1.2 and a p value < 0.05. These spots were found to correspond to 34 proteins by mass spectrometry and Mascote identification (Table I.4). Among the proteins found differentially regulated by TLQP-62, several are mainly related to neurodevelopment and synaptic function (Table I.5). Moreover, some have been linked to neurological disorders in several proteomic studies[289–291]. Proteins found differently expressed by TLQP-62 were analysed with Cytoscape and String tools, with a 0.4 confidence cutoff, showing several of them to be connected and involved in neurodevelopment and synaptic function, with neurogenesis, cell differentiation, cytoskeleton formation, axon growth and guidance, and in energy and glucose metabolism, protein and xenobiotic metabolism, oxidative stress, and immune response (Figure I.9 and Table I.5). Inflammatory pathways and neuronal mechanisms are known to be connected, and alterations in immune and inflammatory processes have been observed in patients with neuropsychiatric disorders, such as depression[292].

DPYSL3, PGAM2, CALD1 and ZNF638 were identified in several different spots, which might indicate different isoforms or post-translational modifications, as glycosylation or phosphorylation. DPYSL3 is necessary for signalling by class 3 semaphorins and remodelling of cytoskeleton, playing a role in axon guidance, neuronal growth, cell migration and oxidative stress and being associated with neurodegeneration[293,294]. DPYSL3 has several serines, tyrosines and threonine that can be phosphorylated by different partners (DPYSL2 and GSK3). PGAM2 has also several threonines, tyrosines and serines available to be phosphorylated and it can also suffer acetylation. This is a glycolytic enzyme with an important role in regulating energy production and biosynthesis of nucleotide precursors and amino acids.

Table I.4 | MASCOTE ANALYSIS OF TLQP-62 EFFECT ON RA-DIFFERENTIATED SH-SY5Y CELLS PROTEOME BY 2D-DIGE.

Spots	Fold	Uniprot Code	Name	kDa	Score
3145	1,4	DPYSL3_HUMAN	Dihydropyrimidinase-related protein 3	61,9	107,7
1361	1,4				116,5
3149	1,3				139,9
1358	1,2				419,4
1361	1,4	SUSD1_HUMAN	Sushi domain containing protein 1	82,7	35,0
3145	1,4	CHD5_HUMAN	Chromodomain helicase DNA binding protein 5	222,9	34,4
1361	1,4	ADD1_HUMAN	Alpha-adducin	80,9	31,3
1352	1,3	ALB_HUMAN	Serum albumin	69,3	226,0
3149	1,3	DPYSL2_HUMAN	Dihydropyrimidinase-related protein 2	62,39	217,8
1357	1,3	NAA20_HUMAN	N-alpha-acetyltransferase 20	20,4	42,7
2658	1,3	PGAM2_HUMAN	Phosphoglycerate mutase 2	28,7	109,1
3152	1,2				128,3
1288	1,3	CALD1_HUMAN	Caldesmon	93,2	77,0
1239	1,2				59,0
3157	1,2	ZNF638_HUMAN	Zinc finger protein 638	220,5	33,9
3146	1,2				33,4
1238	1,2				34,2
3065	1,2				33,8
2662	1,2	PAFAH1B3_HUMAN	Platelet activating factor acetylhydrolase IB subunit γ	25,7	241,5
2725	1,2	UCHL1_HUMAN	Ubiquitin carboxyl terminal hydrolase isozyme L1	24,8	212,1
3157	1,2	GSTP1_HUMAN	Glutathione S transferase P	23,3	341,3
3157	1,2	DYST_HUMAN	Dystonin	860,1	36,2
3157	1,2	AIF1_HUMAN	Allograft inflammatory factor 1	16,7	35,3
2641	1,2	ERP29_HUMAN	Endoplasmatic reticulum resident proetin 29	29,0	310,5
2291	1,2	CNN3_HUMAN	Calponin 3	36,4	347,6
2291	1,2	IDH3A_HUMAN	Isocitrate dehydrogenase NAD subunit alpha	39,6	99,7
2453	1,2	NIT2_HUMAN	Omega amidase NIT2	30,6	247,0
2453	1,2	COPB2_HUMAN	Coatomer subunit beta	102,4	33,7
2107	1,2	MRPS22_HUMAN	28S ribosomal protein S22	41,3	376,8
3159	1,2	PSMB2_HUMAN	Proteasome subunit beta type 2	22,8	87,3
3155	1,2	PSMB3_HUMAN	Proteasome subunit beta type 3	22,9	37,0
2013	1,2	PCBP1_HUMAN	Poly(rC) binding protein 1	37,5	311,2
2013	1,2	HNRPD_HUMAN	Heterogeneous nuclear ribonucleoprotein D0 (AUF1)	38,4	50,9
3155	1,2	PARK7_HUMAN	Protein/ nucleic acid deglycase DJ1	19,9	503,5
1238	1,2	CEP135_HUMAN	Centrosomal protein 135	133,4	34,1
3065	1,2	SOD1_HUMAN	Superoxide dismutase	15,9	355,5
2673	1,2	PNPO_HUMAN	Pyridoxine 5' phosphate oxidase	30,0	33,2
3152	1,2	EIF4H_HUMAN	Eukaryotic translation initiation factor 4H	27,4	90,9
2700	1,2	TPI1_HUMAN	Triosephosphate isomerase	30,8	912,9
2700	1,2	RAN_HUMAN	GTP inducing nuclear protein Ran	24,4	34,2
1624	-1,2	TUBB_HUMAN	Tubulin beta chain	49,6	362,4
1624	-1,2	PDIA1_HUMAN	Protein disulfide isomerase (P4HB)	57,1	176,1

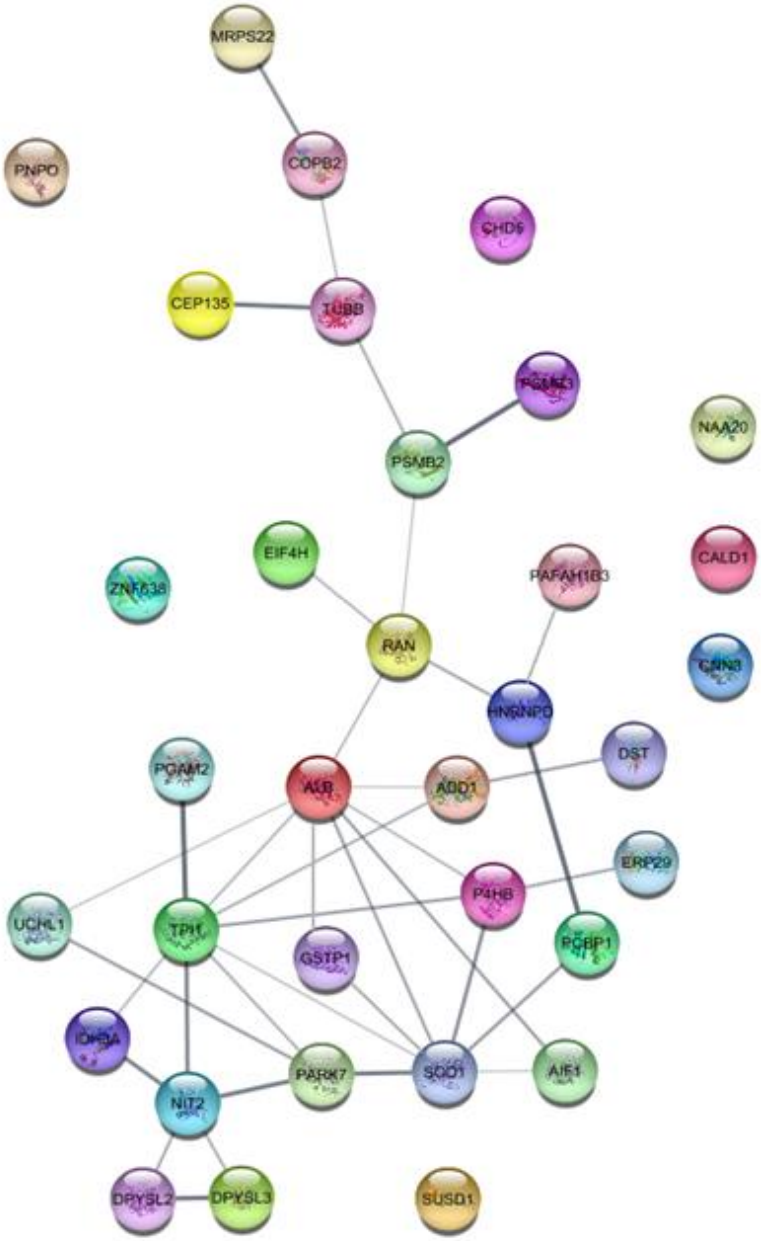

Figure I.9 | INTERACTIONS BETWEEN THE IDENTIFIED PROTEIN FROM 2D-DIGE ANALYSIS USING CYTOSCAPE AND STRING TOOLS.

This protein is stimulated by oxidative stress and regulates cell proliferation[295]. CALD1 has an essential role during cell division and migration during nerve regeneration by regulating microfilament organization as it binds F-actin, as CNN3. This protein also has serine, tyrosine and threonine residues, being phosphorylated by CDK1 and CDK6. ZNF638 is a matrin-like protein working as a transcription cofactor of CEBPs (CCAAT/enhancer-binding *proteins) and a* regulator of adipogenesis[296]. Post-translational modifications includes methylation and phosphorylation. This zinc-finger protein is predicted to be also involved in neurodevelopment.

DPSYL3 showed a 1.4 fold increase in TLQP-62-treated RA-SH-SY5Y cells compared to control, together with SUSD1, CHD5 and ADD1. All these proteins have a role in neurodevelopment. SUSD1 is involved in calcium-ion binding and found to be disrupted in patients with autism and epilepsy. CHD5 is a tumor suppressor expressed in multiple brain regions and highly expressed in differentiated neurons, that regulates neurodevelopmental and emotional processes, being important for dendritic morphology and involved in neurodegenerative and neurodevelopmental disorders[297,298]. This protein is required for neurogenesis and is upregulated in RA-treated SH-SY5Y cells inducing neuronal differentiation through TrkA[299]. ADD1 has been implicated in regulation of adipogenesis[300], but has also a role in neurodevelopment by being a membrane cytoskeletal protein, mediating signal transduction in cellular processes via PKC, PKA and Ca^{2+}/CaM, and regulating actin dynamics[301]. α-adducin is a synaptic protein and so important for synapse formation in hippocampus being present at the synapse, presynaptically and postsynaptically, and is also required for maintaining axon diameter[302,303]. It also interacts with DARPP-32. Moreover, it has been associated with neurodegenerative disorders[304].

Among all the identified protein, several have been associated with neuropsychiatric disorders: DPYSL2, DPYSL3, TUBB, IDH3A, PGAM2, TPI1, ERP29, ALB, SOD1, PARK7 were found differently expressed in brain samples from individuals suffering from schizophrenia compared to controls[291]. Interestingly, as observed in figure I.9, here these proteins were found increase as a response to

Table I.5 | BIOLOGICAL AND NEUROLOGICAL INVOLVEMENT OF IDENTIFIED PROTEINS.

Uniprot Code	Neuronal process	Cellular Process	Associated Neurological Disorder	DISC1 Interaction
ADD1_HUMAN	Neurodevelopment Synaptic Plasticity	Axon growth	SCZD	
AIF1_HUMAN	Neurodevelopment	Immune response Oxidative stress	MDD	
ALBU_HUMAN	Neuronal survival	Xenobiotic metabolism Tryptophan metabolism	BPD, SCZD	
CALD1_HUMAN	Neurodevelopment	Axon guidance Cytoskeleton Cell migration	MDD	
CEP135_HUMAN	Neurodevelopment	Cell cycle		
CHD5_HUMAN	Neurodevelopment Synaptic Plasticity	Neurogenesis Neuronal differentiation		
CNN3_HUMAN	Neurodevelopment Synaptic plasticity	Cytoskeleton Cell cycle		
COPB2_HUMAN	Neurodevelopment	Golgi-ER traffic Protein metabolism		
DPYSL2_HUMAN	Neurodevelopment Synaptic plasticity	Axon growth and guidance Cell signalling	BPD, SCZD, MDD	Yes
DPYSL3_HUMAN	Neurodevelopment	Axon growth and guidance Cell signalling and migration	BPD, SCZD, MDD	Yes
DYST_HUMAN	Neurodevelopment Synaptic Plasticity	Cell signalling Cytoskeleton		Yes
EIF4H_HUMAN	Neurodevelopment	mRNA translation Cell cycle	MDD	
ERP29_HUMAN	Neurodevelopment	Ribosomal assembly	SCZD	
GSTP1_HUMAN	Neurodevelopment	Xenobiotic metabolism Oxidative stress Immune response Steroid metabolism	BPD	
HNRNPD_HUMAN	Neurodevelopment	mRNA metabolism Cell cycle		
IDH3A_HUMAN	Synaptic plasticity	Energy metabolism Protein metabolism	SCZD	
MRPS22_HUMAN		Cell cycle Energy metabolism Protein metabolism		
NAA20_HUMAN	Neurodevelopment	Cell migration Energy metabolism		
NIT2_HUMAN		Xenobiotic metabolism Protein metabolism Fatty acid metabolism Immune response		
PAFAH1B3_HUMAN	Neurodevelopment	Protein assembly ER traffic	BPD	
PARK7_HUMAN	Neurodevelopment Synaptic plasticity	Oxidative stress Energy metabolism	BPD, SCZD	

PCBP1_HUMAN		Immune response Protein metabolism Cell cycle		
PDIA1_HUMAN	Neurodevelopment Synaptic Plasticity	Xenobiotic metabolism Protein metabolism Fatty acid metabolism Oxidative stress		
PGAM2_HUMAN	Neuronal survival	Cell assembly Glucose metabolism Protein metabolism	SCZD	
PNPO_HUMAN	Neurodevelopment	Neurotransmitters metabolism Protein metabolism Fatty acid metabolism		
PSMB2_HUMAN	Neurodevelopment	Immune response Energy metabolism		
PSMB3_HUMAN	Neurodevelopment	Immune response Energy metabolism		
RAN_HUMAN	Neurodevelopment	DNA synthesis Cell cycle		
SOD1_HUMAN	Neurodevelopment Synaptic function	Oxidative stress Energy metabolism Xenobiotic metabolism	BPD, SZCD	
SUSD1_HUMAN	Neurodevelopment	Immune response Cell cycle		
TPI1_HUMAN		Cell growth and communication Energy metabolism Carbon metabolism Steroid biogenesis	SCZD	
TUBB_HUMAN	Neurodevelopment	Cell growth Protein metabolism Energy metabolism DNA replication Cell cycle	BPD, SCZD	Yes
UCHL1_HUMAN	Neurodevelopment Neuron survival Synaptic plasticity	Proteasome degradation		
ZNF638_HUMAN	Neurodevelopment Synaptic plasticity	Insulin secretion		

TLQP-62 effect. Moreover, SOD1 interacts with PARK7, ALB and connects to PARK7, PGAM2 and IDH3A. DPYSL2 and DPYSL3 interact with each other and connect to IDH3A, PARK7 and TPI1 through NIT2, a transcription factor.

In another study DPYSL3, SOD1 and TUBB were found altered in hippocampal samples from subjects with schizophrenia, and DPYSL2, DPYSL3, GSTP1, SOD1, PARK7, PAFAH1B3, TUBB were find altered in hippocampal samples from subjects with bipolar disorder[305].

A study concerning samples from major depressive subjects analysed the differences in phosphorylated proteins compared to normal subjects. The differentially phosphorylated proteins were involved in transport, energy and protein metabolism, cell growth and signalling, and neurogenesis and immunity[306]. GSTP1 plays an important role in detoxification and xenobiotic metabolism, but also in steroid biosynthesis, and is implicated in schizophrenia[307]. PAFAH1B3 is important for brain development in neuronal differentiation[308].

In the present study, GSTP1, SOD1 and PARK7, apparently play a role in neurodifferentiation by regulating oxidative stress. Several proteins involved in energy and glucose metabolism were also identified to be increased in the TLQP-62 treated cells: IDH3A, MRPS22, NAA20, PARK7, PGAM2, PSMB2, PSMB3, SOD1 and TPI1. Some of these proteins also have a role in immune response, cell growth and oxidative stress. Moreover, DPYSL3, CDH5, DST, PARK7, SOD1, GSTP1, RAN and UCHL1 are directly involved in nervous system development process and DPYSL2, DPYSL3, SOD1 and UCHL1 in axogenesis and neurodifferentiation.

5 DISCUSSION

From a clinical point of view adult neurogenesis is highly relevant as its dysregulation can evolve as a significant contributor to neuropsychiatric and neurodegenerative diseases. During neurogenesis new cells suffer migration, axon and dendrite outgrowth and synapse formation to integrate the pre-existing neuronal circuits. Thus, neural progenitor cells proliferation and differentiation, with axogenesis, dendritogenesis and synaptogenesis, are essential processes of neural development, during not only embryogenesis, but through adulthood in the hippocampus.

Understanding the effect and role of TLQP-62 on neuronal cells might help to better understand its role on neurogenesis, proliferation, neurodifferentiation and dendritogenesis, which can also bring some knowledge into chronic mental disorders.

TLQP-62 has been described as having an effect on hippocampal cells neurogenesis and memory, but until very recently, no information about the cell morphological effects of this peptide was available[150,190]. TLQP-62 induces neurogenesis in neural progenitor cells (NPCs) in the adult hippocampus by increasing the number of type2a, rather than the number of more differentiated type3 cells[150]. That study shows TLQP-62 enhances cell proliferation by activating BDNF, phosphorylating TrkB and requiring CaMK, PKD and metabotropic glutamate receptor mGluR5[150]. However, in a recent study TLQP-62 has been shown to be implicated in maturation of hippocampal dendrites, enhancing dendritic branching and outgrowth, and synaptic proteins [149]. TLQP-62 can be further processed into TLQP-21 and AQEE-30, which can potentially have an effect on neurogenesis. AQEE-30 is known to have antidepressant-like effects and TLQP-21 was described as not being required for memory formation but rather to impair fear memory[190]. TLQP-21 has also been reported to have a neuroprotective effect by preventing cell death[252].

In the present study, the effect of TLQP-62 and TLQP-21 on SH-SY5Y neuroblastoma cells morphology was investigated. This cell line is known to express high levels of VGF, which is kept in vesicles in the dendrites. The SH-SY5Y human neuroblastoma cell line was used as it is known to undergo differentiation into a neuron-like cell type in the presence of retinoic acid, with dendrite outgrowth an occasionally connecting with each other. Proteomic analysis was also performed to investigate the effect of TLQP-62 on neuronal cells and the possible signaling pathway in which VGF and TLQP-62 could be implicated, leading to neurogenesis or neurodifferentiation, and more specifically, to neuritogenesis.

5.1 VGF IS REQUIRED FOR NORMAL CELL SURVIVAL AND PROLIFERATION, AND TLQP-62 PROMOTES AND SUPPORTS SH-SY5Y NEURODIFFERENTIATION AND NEURITOGENESIS

TLQP-62 was observed to promote SH-SY5Y neuroblastoma cell differentiation into a more neuron-like morphology type enhancing neuritogenesis, promoting neurite branching and outgrowth. On the other hand, TLQP-21 seems to have no effect on differentiation and moderately decreases proliferation, indicating that the effects seen on neuritogenesis are specific of TLQP-62.

However, VGF-silenced SH-SY5Y have slower proliferative and survival rate, compared to wildtype cells. That could not be rescued by TLQP-62, nor could this peptide induce differentiation of these cells, in the present study. This might indicate that VGF is crucial for normal cell proliferation and survival, but not probably through TLQP-62 or TLQP-21 action. Moreover, these cells fail to respond to TLQP-62, but can undergo differentiation in response to RA, meaning that a higher concentration of TLQP-62 is probably needed for these cells to differentiate or that TLQP-62 uses a different pathway from RA to induce differentiation.

Although TLQP-62 seems to have an effect on SH-SY5Y differentiation, to better illustrate the effect of this neuropeptide would be preferable to use a more neuronal model. Thus, SH-SY5Y cells were first differentiated for 9 days in the presence of RA and treated

with TLQP-62 for 24 h in order to evaluate possible proteomic changes, compared with the RA-differentiated cells.

RA deficiency and abnormal TrkB signaling are involved in the pathophysiology of affective and mood disorders, schizophrenia or Alzheimer's disease, and has been shown to have an effect on adult hippocampal neurogenesis and memory, together with neurotrophins [282,309–312]. RA enters the cell and bind to a retinoic acid receptor (RAR) and retinoid X receptor (RXR) dimer in the nucleus, inducing the expression of several transcription factors. The Trk family plays a crucial role in neuronal survival, differentiation, function, and target organ innervation during development[313]. Neurotrophins and BDNF regulate the expression of their specific Trk receptors. RA, estrogen, interferon γ, cholesterol are capable of inducing Trk expression[314]. This variety of compounds capable of altering Trk expression indicates that diverse signal transduction pathways regulate Trk gene expression. In neurons TrkB is known to be regulated by the cAMP/CREB pathway[315].

SH-SY5Y RA-treatment induces expression of functional TrkA and TrkB-receptors, which expression is lacking on neuroblastoma cells, making cells responsive to BDNF, NGF, NT-3 and NT-4/5[282]. NGF through TrkA, BDNF and NT4 through TrkB, NT3 through TrkC, TrkA or TrkB, can activate 3 difference pathways: PLCy pathway (cell adhesion, migration and synaptic plasticity), PI3K pathway (PI3K phosphorylation leads to the activation of AKT, by PDK, mediating cell survival, growth and proliferation), or MAPK/ERK pathway (Ras phosphorylation activates Raf/MEK/ERK/ S6K1 pathway leading to phosphorylation of transcription factors implicated in the activation of genes involved in differentiation, maturation and neurite growth)[270]. Pro-neurotrophins bind to p75NTR activating JunK1 pathway and p53 pathway leading to cell death, or promote cell survival, neurite outgrowth and cell migration, through NF-κB[316]. Neurotrophins regulate cell fate decisions, axon growth, dendrite growth and the expression of proteins, such as ion channels, transmitter biosynthetic enzymes and neuropeptide transmitters that are essential for normal neuronal function, like VGF. Continued presence of the neurotrophins is required in the adult nervous system,

where they control synaptic function and plasticity, and neuronal survival, morphology and differentiation.

It has been described that adding BDNF to RA-induced SH-SY5Y cells supports and promotes neuritogenesis and synaptogenesis[283–286]. On the other hand, in the present study TLQP-62 shows to induce neuritogenesis in SH-SY5Y cells and to support RA-induced differentiation, as cells survive and continue to differentiate in the presence of TLQP-62 and absence of RA. This suggests that TLQP-62 might not only lead to the expression of BDNF, but also of TrkB receptors, considering that in this cell line the TrkB expression is downregulated. VGF has been demonstrated to be regulated by antidepressants in hippocampus, and the BDNF/TrkB/CREB and monoamide transmitter pathways mediate antidepressant effects. BDNF/TrkB signalling has also been related to obesity and nociception, learning and memory, and hippocampal function[136,317–319]. TLQP-62 has been showed to increase BDNF expression and TrkB/CREB phosphorylation independently of serotonin or dopamine but requiring glutamate[150,190,251]. Moreover, TrkB blocking almost completely abolished TLQP62-induced antidepressant effects, BDNF upregulation, and CREB/TrkB phosphorylation[251]. Moreover, Cyclin-dependent kinase 5 (CDK5) is involved in BDNF/TrkB-stimulated dendritic growth in hippocampal neurons through Rho GTPase Cdc42 activation, which can lead to PI3K/AKT activation which inhibits GSK3β[284,320]. Cdc42 regulates signalling pathways that control cell morphology, cell migration, endocytosis and cell cycle progression. Rho GTPases are central to dynamic actin cytoskeletal assembly and rearrangement that are the basis of cell-cell adhesion and migration. Activated Cdc42 activates by conformational changes p21-activated kinases PAK1 and PAK2, which in turn initiate actin reorganization and regulate cell adhesion, migration, and invasion[321].

TLQP-62 induces and is induced by BDNF, indicating a regulation loop. TLQP-62 might, moreover, participate directly in the binding BDNF/TrkB, as no receptor for this peptide could be identified so far. However, TLQP-62 might have other receptor(s), depending on the tissue and function, which can result in the

activation of different signaling pathways, leading to the expression of BDNF and TrkB.

5.2 TLQP-62 AND DISC1 REGULATES NEURONAL DIFFERENTIATION

VGF-silenced and DISC1-silenced SH-SY5Y neuroblastoma cells were used as a model to evaluate its effects on RA-induced neurodifferentiation of those cells, in an attempt to determine which converging signaling pathways connecting DISC1 and VGF/TLQP-62 could be implicated in neurodifferentiation and possible in chronic mental disorders.

Lack of VGF originates rounder cells, with a shorter life-time and with a slower response to RA-differentiation. Also, DISC1 silencing causes a deficit in neurite outgrowth with a reduction in number and size of neurites. Thus, indicating there is a connection between DISC and VGF, and not only DISC1 but also VGF and TLQP-62 have an important and fundamental role in neurodifferentiation and neuritogenesis, being capable of support the effects caused by DISC1 silencing or DISC1 overexpression.

RA is known to induce TrkB receptor making SH-SY5Y responsive to BDNF[282], and from the present study, also to TLQP-62. BDNF activates the PI3K/AKT pathway and induces VGF, and its inhibition causes neurite outgrow to fail in the presence of RA. If TLQP-62 is capable of induce differentiation, it is possible that it induces TrkB, somehow. If both DISC1 and VGF silencing causes abnormal neurite outgrowth means that both proteins are needed to mediate this process.

DISC1 interacts with many other proteins involved in different signalling pathways: GSK3β enhances neural proliferation, through PI3K/AKT/mTOR pathway, and NDEL1/LIS1 regulates neuronal migration[146,271]. DISC1 seems to indirectly regulate VGF expression through the PI3K/AKT/CREB pathway[148]. That same pathway is induced by BDNF and upregulates VGF. DISC1 might be important for proper regulation of VGF levels and to maintain a regulatory loop between VGF and BDNF during differentiation.

5.3 TLQP-62 SUPPORTS NEURITOGENESIS BY INDUCING AND SUPPORTING THE EXPRESSION OF SEVERAL PROTEINS INVOLVED IN NEURODEVELOPMENTAL AND SYNAPTIC PROCESSES

To unveil the molecular effects of RA and TLQP-62 on these cells, and investigate which signaling pathways could increase the expression of BDNF and TrkB, a proteomic study was performed. Several proteins involved in fundamental cellular processes are increased in response to TLQP-62 in RA-differentiated SH-SY5Y cells.

Neurogenesis is a neurodevelopment stage involving NPCs proliferation, migration and neuron differentiation with integration in the pre-existing neuronal circuits. Dendritogenesis, with spine morphogenesis and dendritic arborisation, is a crucial process for the establishment of synaptic plasticity, allowing neurons communication. During neurodevelopmental events several proteins related to cell cycle regulation, cytoskeleton organization and axon growth are overexpressed to prepare and support neurons for those processes.

Differences regarding metabolic requirements between proliferative and differentiated cells have been reported[322,323]. Neurons have high energetic requirements due to the repeated generation of postsynaptic and action potentials, and neurotransmitters cycle[324,325]. Thus, neurons have a high activity of glucose and lipid metabolism and oxidative stress. Also, the biosynthetic pathways are essential during neuron proliferation and differentiation. The mitochondrion plays a fundamental role in neurogenesis, in neuroplasticity and neurodifferentiation, in axon and dendritic growth and synapse formation with neurotransmitters release. CREB and BDNF are involved in synaptic plasticity and enhance mitochondrial energy production. Also, mTOR has been linked to the regulation of metabolic pathways in neuron maturation.

Among the identified proteins several are somehow involved in neurodevelopment or synaptic function, with neurogenesis, cell differentiation, cytoskeleton formation, axon growth and guidance, and in cell cycle and growth and communication (Figure I.10), but also in energy and glucose metabolism and metabolites biosynthesis, and oxidative stress and immune response (Figure I.11).

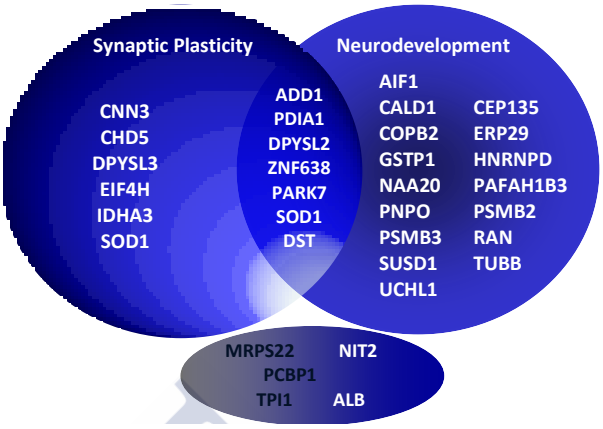

FIGURE I.10 | PROTEINS INVOLVED IN NEURODEVELOPMENT OR SYNAPTIC PLASTICITY.

Analysing the set of identified proteins, using the Cytoscape and String tools, several other proteins appear related to them. Among them are GSK3β, CDK1 and p53, involved in DNA repair and cell cycle regulation during development. GSK3β is a kinase, which increased expression is associated with bipolar disorder, and is inhibited by ERK1/2. This protein is involved in energy metabolism and neurodevelopment. CDK1, is a kinase that phosphorylates several substrates involved in cell cycle progression. Tumor protein p53 is a tumor suppressor playing a role in apoptosis and genomic stability, activating the expression of several genes implicated in cell cycle.

RA promotes neurogenesis and neurodifferentiation by being involved in the switch between proliferation and differentiation. This effect is thought to occur via retinoic acid receptors and Wnt signalling pathway. RA induces several changes in the cell and promotes the expression of several genes involved in energy metabolism preparing the cell for changes in energy requirements and cellular morphology associated with neurogenesis by adjusting lipid content[326,327]. RA induces CDK1 that interacts with the retinoid acid receptor γ (RARγ) modulating the levels of AKT and $P27^{kip}$ phosphorylation influencing cell cycle progression and differentiation[328].

SH-SY5Y cells were induced by RA and differentiated into a more neuron-like phenotype. TLQP-62 showed to be able to support and maintain those cells differentiation, promoting neuritogenesis, through regulating the expression of several proteins involved in cytoskeleton organization and cell cycle regulation. The majority f the identified proteins play a role in cell cycle regulation and several are responsible for arresting cell cycle, inhibiting cell proliferation.

The generation, migration, and differentiation of neurons require the functional integrity of the microtubule cytoskeleton. ADD1 is induced by RA and phosphorylated by CDK1 contributing for cytoskeleton and cell–cell adhesion and to maintain axon diameter[301]. It mediates signal transduction in cellular processes via PKC, PKA and CaMKII, and regulates actin dynamics[301]. α-adducin is present at the synapse, presynaptically and postsynaptically[302,303]. Moreover, it has been implicated in the regulation of adipogenesis and with neurodegenerative disorders[304]. ADD1 is inhibited by GSK3β, and so an increase in ADD1 might indicate that GSK3β is inhibited in the presence of TLQP-62. Moreover, GSK3β inhibition enhances RARγ activity an cell differentiation[329,330]. CALD1 is regulated by CDK1 and CaMKII binding and phosphorylation, modulating cell shape, cytokinesis, cell adhesion, having an important role in regulating cell morphology and motility. CALD1 regulates microfilament organization and interacts with actin and CNN3. CNN3 is found expressed in the brain and may regulate actin cytoskeleton formation with a role on neural plasticity. CNN3 is also found in dendritic spines of adult hippocampal neurons being crucial for central nervous system development[331]. The overexpression of this protein induces neural differentiation by regulating ionotropic glutamate receptors, GluR1 and NR1[332]. CNN3 has been suggested as an adaptor protein in PKC and ERK signalling. CEP135 is a centrosomal protein and a microtubule-associated protein (MAP) involved in microtubule organization, important for cell cycle regulation[333]. Its downregulation causes microcephaly and disturbed centrosomal function[334,335]. It binds to tubulin to stabilize and destabilize microtubules, guides it towards specific cellular locations, crosslink microtubules and mediates interactions of microtubules with other proteins in the cell.

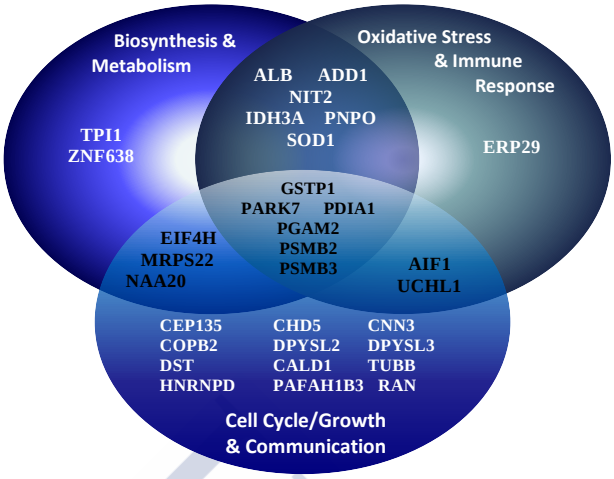

FIGURE I.11 | CLASSIFICATION OF THE IDENTIFIED PROTEINS ACCORDING TO THEIR CELLULAR AND MOLECULAR FUNCTIONS.

Surprisingly, TUBB was found downregulated in this study. TUBB is an important protein for normal brain structure, being highly expressed in the developing cortex[336]. Perturbation of TUBB can alter the mitotic index of progenitor cells and their subsequent migration. Depletion of TUBB causes alterations in cell morphology, dendritic spine density, neuronal complexity and axon outgrowth in the mouse cerebral cortex, by altering the dynamic properties of the microtubule cytoskeleton during neurodifferentiation[337].

NAA20 is the catalytic subunit of the NatB complex which acetylates specific proteins and is required for maintaining the structure and function of actomyosin fibers, which are implicated in cell signaling pathways, including Hippo, Ras-MAPK, PI3K and NF-kB, that are critical in the control of cell cycle progression (disruption of actin filaments causes G1 arrest and impaired cytokinesis), cell growth, cell size and cell motility[338,339]. HNRNPD (or AUF1) is a nucleic acid binding protein that regulates mRNA stability and interacts with other proteins in developing cortical neurons. It is specifically expressed in subsets of proliferating neural precursors and differentiating postmitotic neurons of the developing cerebral cortex[340,341].

DPYSL2 is a risk gene for schizophrenia and responds to mTOR, involved in CNS development, neuronal growth and proliferation[342,343]. Perturbation of mTOR signaling affects neurotransmitters, as serotonin, glutamate and their receptors. DPYSL2 is also involve in axonal growth and is inhibited by RA during SH-SY5Y differentiation. DPYSL2 is regulated by transcription factor AP2 and Pax3. In this study DPYSL2 is upregulated by TLQP-62. DPYSL3 levels increases during RA-induced cell differentiation. DPYSL2 and DPYSL3 are both involved in neuronal polarity and axon elongation, are phosphorylated by CDK5, and are known to interact with DISC1[344]. GSK3β phosphorylates DPYSL2 and DPYSL3, through which controls mitotic chromosomal alignment. DST is large protein of the cytoskeletal filament that interacts with MAP1B and clathrin, being essential for neuronal cytoskeleton organization[345].

PAFAH1B3 is important for brain development in neuronal differentiation and migration[308]. PAFAH1B3 interacts and forms a phospholipase complex with PAFAH1B2 and PAFAH1B1/LIS1 which regulates the functional organization of Golgi complex[308,346,347]. LIS1 regulates the motor protein dynein and interacts with DISC1. COPB2 is part of the cytosolic Golgi coatomer complex constituting the coat of nonclathrin-coated vesicles which is essential for Golgi budding and vesicular trafficking. This protein has been associated with congenital brain malformation and to be crucial for neurodevelopment [348]. COPB2 has been described as suppressing cell proliferation by regulating cell cycle-related protein and arresting cell cycle[349].

RAN GTPase is involved in the transport of RNA and proteins into and out of the nucleus during interphase and mitosis. It is also involved in DNA synthesis and is a central regulator of cell cycle progression[350]. Ran, together with its auxiliary factor, Ran GTPase activating protein 1, have a role during cell differentiation[351]. RA and NGF induces CHD5 upregulation, a tumor suppressor gene and chromatin remodeler, regulating neuronal differentiation through TrkA receptor[299]. CDH5 is phosphorylated by CDK1 and binds and represses the G2/M checkpoint gene WEE1. CHD5 is required for

neurogenesis and dendritic morphology associating and facilitating gene activation and expression of a large set of genes required for neuronal genes activation. CHD5 binds to histone 3K27 required for neurogenesis and facilitates the repression of genes involved in gene repression regulation[297,298]. CHD5 might promote P53 activation leading to tumor suppression.

EIF4H is a translation initiation factor that regulates protein synthesis at the synapse, under p65/NF-kβ control, during cell proliferation or differentiation. In a mouse model, EIF4H knockout leads to smaller brain volume individuals with reduction of number and complexity of neurons with impair in memory and learning[352]. ERP29 is a protein disulphide isomerase and molecular chaperone showing a tumor suppressive activity and regulates cell survival, by modulating PI3K/AKT and β-catenin[353,354]. It regulates CDK inhibitors arresting cell cycle. ERP29 upregulation protects axotomized neurons from apoptosis, prevents reduction of number of neurons and reduction of neurite length, and promotes regeneration associated with ERK and PI3K signal[355]. In another study, cortical neurons with upregulated ERP29 showed increased proliferation and axon outgrowth with increased PKC[356]. There is no evidence that retinoic acid promotes ERP29 expression, and so its increasing is the present study is probably due to TLQP-62 action. ERP29 strongly binds to PDIA1. PDIA1 is a multifunctional protein found in ER with chaperone functions and roles in cell migration, cytoskeletal regulation and organization, and thiol redox signalling that prevents neuronal death during ER stress. This protein is downregulated in diabetes and its dysregulation causes disruption of motorneuron connectivity[357,358]. Surprisingly, in the present study PDIA1 was found downregulated.

Thus, some of the proteins involved in cell cycle regulation and cytoskeleton organization, have also a role in oxidative stress and immune response. Increased oxidative stress decreases BDNF levels in individuals suffering from bipolar disorder[359]. Adult neurogenesis is critical for brain function, and reactive oxygen species (ROS) accumulate during this process as a physiological mechanism, increasing oxidative stress[360,361]. It has also been described changes

in cell cycle and up-regulation of neuronal markers during SH-SY-5Y neurodifferentiation by retinoic acid mediated by oxidative stress[362]. In those cells oxidative stress is apparently modulated by mTORC1, preventing cell death[363,364].

ALB has been found downregulated by all-trans-RA, but was found increased in this study[365]. Albumin and IgG have been associated with neurological disorders[366,367]. AIF1, an inflammatory protein, modulates proliferation and is upregulated by RA[368]. GSTP1 has been showed to be downregulated by RA through RARβ and increased by insulin[369]. From the present study, GSTP1 is induced by TLQP-62. GSTP1 is phosphorylated and enhanced by PKA and PKC, and arrests cells in G0/G1 phase by upregulating p21 expression[370]. High levels of GSTP1 inhibits cell proliferation by reducing AKT, which in turn phosphorylates GSTP1, forming a regulatory loop[371,372]. GSTP1 plays an important role in detoxification and xenobiotic metabolism, but also in steroid biosynthesis, and is implicated in schizophrenia[307]. PARK7 is involved in several cellular processes and in signal transduction, being capable of activate ERK1/2 pathway and PI3K/AKT pathway to mediate cell survival and proliferation, attenuating cell death pathways. Moreover, PARK7 helps protecting cells, particularly brain cells, from oxidative stress, and acts as a chaperone molecule and may assist in delivering selected proteins to proteasomes. It is associated with Parkinson disorder and schizophrenia, together with UCHL1, also upregulated in this present study[373]. UCHL1 is mainly expressed in brain and is involved in neurodegenerative disorders. It is a key enzyme in degradation pathway, associated with cytoskeleton organization and protects cells from oxidative stress, allowing cell survival[374]. SOD1 protects cells against oxidative stress, and plays a critical role in proliferation and neurogenesis, being increase during these processes. It also regulates amino acid biosynthesis and monoaminergic neurotransmission and has been found downregulated in schizophrenia [375,376]. SUSD1 is involved in calcium-ion binding and found to be disrupted in patients with autism and epilepsy.

Some other proteins are involved in the energy metabolism and cellular biosynthesis necessary to proper cell functioning and survival, and during neurodifferentiation. Proteins involved in insulin, steroid and cholesterol biosynthesis and metabolism, in carbon and fatty acid metabolism, in glucose metabolism, and in neurotransmitters synthesis and metabolism were found increased.

TPI1 is involved in glycolysis, an energy source process which produce ATP, NADPH, fatty acids, amino acids and nucleotides. An increase of this protein expression happens during cell growth or oxidative stress. IDH3A promotes ATP production and couples mitochondrial metabolism to synaptic transmission protecting cell from oxidative stress and has been implicated in neuropsychiatric disorders[377]. Abnormal IDH3A levels may disrupt mitochondrial function and contribute to the pathogenesis of these disorders. Downregulation of IDH3A causes reduction of the metabolite α-ketoglutarate (αKG) and defects in synaptic transmission. This metabolite enhances synaptotagmin 1 (Syt1) which promotes fusion of synaptic vesicles and neurotransmission[377]. NIT2 has an omega-amidase activity to remove potentially toxic intermediates by converting α-ketoglutaramate to α-KG. Overexpression arrests cells in the cell cycle G2 phase.

PGAM2 is a phosphoglycerate mutase involved in the glycolytic pathway and may contribute to the biosynthesis of amino acids, 5-carbon sugar, and nucleotides precursors. It is stimulated by SIRT2 during oxidative stress and the same mechanisms that induce PGAM2 changes gene expression of mitochondrial proteins probably through mTOR signalling pathway[295,378]. Mitochondrial dysfunction have been associated with schizophrenia[126]. MRPS22 is a mitochondrial ribosomal protein that helps in protein synthesis within the mitochondrion. PSMB2 and PSMB3 are part of a proteasome which recognizes degradable proteins, including damaged proteins for protein quality control purpose or key regulatory protein components dynamic biological processes, as cell growth and differentiation, gene transcription, signal transduction and apoptosis.

Moreover, several of the identified proteins have been associated with neuropsychiatric disorders, as is the case of ALB, DPYSL2, DPYSL3, ERP29, GSTP1, IDH3A, PAFAH1B3, PARK7, PGAM2, SOD1, TPI1 or TUBB, which have been found altered and decreased in samples from subjects with schizophrenia and bipolar disorder[291,305].

Thus, TLQP-62 induces and supports RA-induced changes on differentiating cells, regulating several proteins involved in cellular processes crucial for cell differentiation and survival. Several of those proteins are involved in cell cycle regulation, inducing cell cycle arresting and inhibiting proliferation; some other proteins are involved in oxidative stress and immune response, allowing cell protection and survival; and other proteins are involved in energy metabolism and biosynthesis and in cytoskeletal organization, with axon and dendrite outgrowth, allowing neurodifferentiation. All those proteins contribute somehow for neurodevelopment and synaptic plasticity processes.

Among those proteins there are several kinases and transcription factors involved, which lead to the upregulation and activation through phosphorylation of several other proteins involved in different cellular processes, but to better understand the downstream mechanisms induced by TLQP-62 it is crucial to unveil its receptor in neuronal cells.

For future work, also a phosphoproteomic study would be of great interest, to help understand the signalling pathways involved.

CHAPTER II

Olfactory Receptor 5P3:

A human TLQP-62 receptor

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CHAPTER II

Olfactory Receptor 5P3: a human TLQP-62 receptor

1 INTRODUCTION

Of all the VGF derived peptides, TLQP-21 has been the only one to have possible cell surface receptors identified. Chen *et al.* identified gC1qR, showing that TLQP-21 activated rat macrophages through gC1qR, causing mechanical hypersensitivity in rats[232]. gC1qR protein was originally described as the receptor to the globular domains of the first complement component C1q and is expressed by both brain and spinal cord derived microglia being indispensable for adipogenesis and insulin signaling[296]. Hannedouche *et al*. identified the complement receptor C3A receptor-1 (C3AR1) as a receptor for TLQP-21[379]. C3AR1 is a G-protein coupled receptor originally thought to be restricted to the innate immune response. More recently, in our group Akhter *et al.* described heat shock protein HSPA8 as a binding partner and potential receptor for TLQP-21[380]. The discovery of these receptors will help identify the mechanisms by which TLQP-21 and possible other derived peptide may modulate its actions.

The most accepted hypothesis is that there are different receptors for the different VG-derived peptides. To date, no TLQP-62 receptors have been described. Since this peptide has an important role in neuronal processes, the identification of its receptor(s) could help better understand the downstream mechanisms and signaling pathways involved in TLQP-62 effects. VGF mRNA is widely expressed in neurons throughout the brain in central system and in peripheral endocrine and neuroendocrine tissues. In the adult brain VGF mRNA has the highest expression in the hypothalamus and the cerebellum, but it is also expressed in cortex, hippocampus, basal ganglia, thalamus, amygdala, midbrain, the main and accessory olfactory bulbs, and the brainstem. VGF and its derived peptides are

found in dense core vesicles and are released in response to depolarizing signals from neuronal and neuroendocrine cells through the regulated secretory pathway. VGF-derived peptides are prominent in the adult spinal cord, in motor neurons of the ventral horn and in the dorsal horn neurons. TLQP peptides are expressed at higher levels in ventral hippocampus and plasma, when compared to other VGF-derived peptides[221].

A receptor is a protein on a given cell that after binding a ligand promotes a biological response and signal transduction. Receptors can be localized on cell surface membrane or intracellularly in the cytosol or nucleus. To isolate and identify a given receptor is necessary to know if the ligand in study is biological active and has a cellular function. Then, the tissue or cell line used in the experiment should express high levels of the ligand, since his might indicate also high expression levels of the receptor, increasing the chances of getting ligand-receptor complex. The next step is isolate cell membrane fraction and cytosolic fraction. Membrane fraction should be solubilized in order to facilitate ligand-receptor interaction and further purify this complex. The known ligand can be chemically, biologically or radiologically labelled. Fluorescently labelled ligands are widely used has it allows fluorescent confocal microscopy. Biotinylated peptides are also commonly used in peptide-receptor interaction, as it allows peptide-receptor complex isolation in an avidin column by affinity chromatography. To increase peptide-receptor binding, a chemical covalent crosslinker can be used in order to facilitate the complex purification and further identification by mass spectrometry.

2 OBJECTIVE

The main objective of this chapter and study is to identify a potential human receptor for the human VGF-derived peptide TLQP-62, using SH-SY5Y cell line and human hippocampus, by covalent crosslinking of the biotinylated peptide with the potential receptor and affinity chromatography followed by mass spectrometry analysis of the isolated complex.

3 METHODS

3.1 HUMAN HIPPOCAMPUS HOMOGENATE PREPARATION

Human hippocampus was obtained from an archive collection approved by the Ethics Review panel of Xunta de Galicia (Spain) and performed in accordance with the ethical guidelines of the Helsinki Declaration. Homogenate from 1 g hippocampus was prepared adding 10 mL PBS with 20% glycerol, 0.1% Triton X-100, 100 µM PMSF, 1 mM EDTA and 1 mM DTT. The homogenate was centrifuged for 10 minutes at 4ºC, 1000xg, pellet was kept frozen and supernatant was collected for further centrifugation for 30 minutes at 100 000xg. Supernatant was discarded and pellet was resuspended in PBS with 20% glycerol and 4% n-octyl-β-D-glucopyranoside and further centrifuged for 30 minutes at 100 000xg. Supernatant (S), containing the cytosolic fraction, and pellet (P), containing the membrane fraction, were collected for protein precipitation and quantification.

3.2 SH-SY5Y CELL CULTURE & PROTEIN FRACTION PREPARATION

SH-SY5Y cells were maintained in a 1:1 proportion EBSS and F12HAM medium, supplemented with 15% FBS, 1% L-glutamine 200 mM, 1% MEM-NEAA and 1% Penicillin/Streptomycin. Cells were seeded at a density of 10^4 cells/ cm^2 in 100x20 mm culture dishes (Falcon, Life Sciences) and grown at 37ºC in a 5% CO_2 humidified incubator. Confluent SH-SY5Y cell plates were washed twice with cold PBS and solubilized in lysis buffer (20 mM HEPES pH 7.4, 2 mM EGTA, 1 mM DTT, 1 mM sodium orthovanadate, 1% Triton X-100, 10% glycerol, 2 µM leupeptin, 400 µM PMSF, 50 µM β-glycerophosphate and 100 µg/ml aprotinin). The cells were scrapped on ice for 10 min and incubated on ice for 30 min with periodic vortexing at each 10 min. Sonication on ice was performed for 3 periods of 15 seconds with 10 seconds interval on ice in between each pulse, with a 10% amplitude. Centrifugation at 4ºC, 14000x*g*, for 30 min was performed. Pellet was discard and supernatant was recovered

and 1 mM DTT and 2% n-octyl-β-D-glucopyranoside was added for membrane protein solubilization, and briefly sonicated for protein precipitation and quantification (see below), and kept frozen at -80ºC.

3.3 PROTEIN PREPARATION & QUANTIFICATION

Protein fractions were precipitated in 6 volumes of cold methanol 100% for 45 min at -20ºC, followed by centrifugation at 4ºC, 14000x*g* for 20 min. Supernatant was discarded and pellet was let to air-dry and resuspended in 50 µL sample buffer (PBS with 1% n-octyl-β-D-glucopyranoside, 10 mM DTT, 1 mM EDTA, 100 µM PMSF) and sonicated for 5 min in a ultrasonic cell disruptor Sonifier 150 (Branson) for solubilization. Protein quantification was performed with Protein Reagent (Bio-rad) as described in chapter I.3.4.

3.4 BIOTINYLATED TLQP-62 PEPTIDE & CROSSLINKER CONJUGATION

Human biotinylated TLQP-62 with an extra cysteine residue with total molecular weight 7833 Da was purchased from ChinaPeptides Co. Ltd, Sanghai, at >95% purity, confirmed by HPLC and MS analysis, as a lyophilized power and stored at -80ºC. Biotin-TLQP-62 was prepared at 1 mg/mL (128 µM) in 20% acetonitrile in filtered PBS with 50 µM TCEP and used or kept at - 80ºC. The peptide sequence is **biotin-CTLQPPSALRRRHYHHALPPSRHYPGREAQARRAQEEAEAEERRLQEQEELENYIEHVLLRRP**.

Sulfo-EMCS (sulfo-N-[ε-maleimidocaproyloxy]succinimide ester) was used as a cross-linker to bind TLQP-62 to its putative receptor. 1 mM Sulfo-EMCS (Thermo Scientific) was dissolved in conjugation buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2 [PBS]) and mixed with 50 µM biotin-TLQP-62 and incubated on ice for 2 hours for peptide conjugation. For the crosslinking reaction the conjugated peptide (1 mL at 50 µM) was mixed with *SH-SY5Y cell homogenate* fraction (1 mL at 2 mg/ml) or *human hippocampus homogenate fraction* (1 mL at 2 mg/mL) and incubated on ice for 2 hours. The reaction was terminated by adding 50 mM Tris-HCl, pH 8.0. As control, cell homogenate, unconjugated sulfo-EMCS and conjugation buffer, without conjugated peptide were incubated. Samples were then submitted to an avidin agarose column for further protein purification and identification as a putative receptor of TLQP-62.

3.5 MONOMERIC AVIDIN AGAROSE AFFINITY CHROMATOGRAPHY

Pierce Monomeric avidin kit (Thermo Scientific) was used for affinity chromatography using a 2 mL prepacked monomeric 4% beaded avidin column, **wash buffer** (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, with 0.01% sodium azide), **biotin blocking/elution buffer** (PBS with 2 mM D-biotin) and **regeneration buffer** (0.1 M glycine, pH 2.8). All components were stored at 4ºC and equilibrated to room temperature before use. Columns were washed with 8 mL wash buffer followed by 6 mL biotin blocking/elution buffer to block non-reversible biotin binding sites. Regeneration buffer was added to remove biotin from reversible binding sites followed by 8 mL wash buffer. Biotinylated proteins should be added to the column and incubated, then washed, eluted and finally regenerated by washing with 4 mL regeneration buffer to strip off residual bound biotin and then washed with 5 mL PBS with 0.01% sodium azide and stored at 4ºC. All fractions were precipitated and pellet was resuspended in 1x Laemmeli buffer for SDS-PAGE and western blot analysis.

3.5.1. Purification of biotin-TLQP-62 crosslinked with SH-SY5Y membrane fraction

SH-SY5Y cells were grown as described before until confluence. Culture medium was removed; cells were washed 3 times with cold PBS and incubated with 2 mL PBS containing 1 mM sulfo-EMCS with 50 µM biotin-TLQP-62 for 2 h at room temperature. Cells were lysed and membrane fraction (P) was recovered as described before. 2 mL of solubilized biotinylated SH-SY5Y membrane proteins were applied to the avidin column and incubated 1 hour at room temperature. Column was washed with 12 mL wash buffer and eluted with 10 mL biotin blocking/elution buffer, collecting fractions for further precipitation and analysis by SDS-PAGE and SYPRO® Ruby staining and Western blot. As control, cell homogenate with1 mM sulfo-EMCS but without biotin-TLQP-62, was added to a column under the same conditions and analyzed.

3.5.2. Purification of biotin-TLQP-62 crosslinked with hippocampus homogenate proteins

Hippocampus homogenate was prepared as described before for cytosolic (S) and membrane (P) fractions. To 1 mM sulfo-EMCS was

added 50 µM biotin-TLQP-62 and let for 2 h at room temperature, before adding P or S hippocampus fraction for 2 more hours. Resulting sulfo-EMCS-biotin-TLQP-62 was applied to the avidin column and incubated 1 hour at room temperature. Column was washed with 12 mL wash buffer and eluted with 10 mL biotin blocking/elution buffer, collecting fractions for further precipitation and analysis by SDS-PAGE and SYPRO® ruby staining and Western blot. As control, hippocampus fraction with1 mM sulfo-EMCS but without biotin-TLQP-62, was added to a column under the same conditions and analyzed.

3.6 MONODIMENSIONAL (1D) SDS-PAGE and SYPRO® RUBY STAIN

Protein extracts or eluted fractions were submitted to SDS-PAGE and SYPRO ruby gel stain as described in chapters I.3.5 and I.3.8

3.7 IMMUNOBLOTTING

Immunobloting was performed as described in chapter I.3.6. Primary and secondary antibodies were diluted in 1% BSA in TBS-T. Goat anti-human HSPA8 (Invitrogen; 1:1000 dilution) and anti-OR5P3 (Invitrogen; 1:1000 dilution) were used as primary antibodies. Secondary antibody anti-goat IgG-HRP (Santa Cruz; 1:2000 dilution).

3.8 MASS SPECTROMETRY

Bands chosen for analysis, after SYPRO Ruby gel staining and visualization, were excised from the gel using a Blue Box (Invitrogen) to aid visualization and sent for protein identification by nLC-ESI-trampa with tryptic digestion and Mascot analysis at Mass Spectrometry Unit at *CACTUS*, University of Santiago de Compostela, as descried before in chapter I.3.9.

3.9 IMMUNOCYTOCHEMISTRY

SH-SY5Y cells were plated and grown, as described before, in a 4-well Milicell EZ slide (Millipore). At the next day cells were washed twice with PBS and fixed with 10% formalin. For blocking, cells were washed with PBS for 5 min and incubated with 2% BSA in PBS for 30 min. Primary antibodies against the human proteins (rabbit anti-VGF(C-term), anti-HSPA8 and anti-OR5P3) were diluted in PBS

according to manufacturer instructions and added to the cells for 1 hour at room temperature. Cells were washed 3 times for 5 min with PBS before incubated with the secondary antibody donkey anti-rabbit IgG-FITC (Santa Cruz Biotechnology) in PBS for 30 min and rinsed with PBS 3 times for 5 min. DAPI was added to the cells (1:1000 in PBS) for 5 min and rinsed with PBS for 5 min, twice. Cells were observed using an Olympus inverted microscope IX51 with an Olympus U-RFL-T reflected fluorescence system and the microscope imaging software Olympus CellSens standard.

3.10 ANALYSIS OF HSPA8-TLQP-62 INTERACTION BY DYNAMIC MASS REDISTRIBUTION ANALYSIS

Label-free high sensitivity plates (PerkinElmer 6057460) were activated with 15 µl of 400 mM N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) (Sigma-Aldrich) and 100 mM Sulfo-N-hydroxysulfosuccidime (sulfo-NHS) (Ther moFisher) diluted in ultrapure water 30 min at room temperature. Microplates were subsequently washed four times with ultrapure water.

HSPA8 immobilization was performed by adding 15 µl of 25 µg/ml of protein in 20 mM sodium acetate buffer at pH = 5. After overnight incubation at 4°C, microplates were washed four times with PBS containing 0,005% Tween-20 buffer, pH = 7.4. Baseline was read after the microplate was equilibrated inside the EnSpire® Multimode Plate Reader (Perkin Elmer) for 3 hours.

TLQP-62 peptide dilutions were prepared in PBS containing 0,005% Tween-20 buffer, pH = 7.4. To this, 15 µl of peptide solution were added to the plate and mixed. Final reading was performed every two minutes over a period of 1 hour.

3.11 ANALYSIS OF OR5P3-TLQP-62 INTERACTION BY CAMP MEASUREMENT

In order to evaluate the effect of TLQP-62 on the activation of OR5P3 it was necessary to generate a cell model overexpressing this olfactory receptor to them measure cAMP levels. This experiment was performed in collaboration with BioFarma Group and Dr. Eduardo Dominguez at Universidad de Santiago de Compostela, Spain.

3.11.1. Hana3A cells and culture media

Hana3A cells derived from HEK293T have the specific characteristic of expressing genes important for the expression and functionality of olfactory receptors (i.e. RTP1, RTP2, REEP1 and Golf). Cells were grown in DMEM (Gibco) with 10% FBS (Sigma Aldrich), 1% P/S (Sigma Aldrich), 2.5% puromycin (Sigma Aldrich) at 37ºC with 5% CO_2.

3.11.2. Recombinant expression of OR5P3

For the recombinant expression of OR5P3 the plasmid pCI-OR5P3 was used. For the genetic transfer of the receptor to the HANA3A cells the FuGENE6 protocol (Promega) was used following the fabricant recommendations. A 3:1 ratio of cDNA:FuGENE6 was used to incubate 15000 cells/well in a 96 well plate. The minimum media OptiMEM (Gibco) was used to optimize the complex formation and cells were incubated for 24h.

3.11.3. Immunofluorescent detection of OR5P3 expression

Cells were permeabilized with HBSS (Gibco), PBS, 2% PFA, 1% Saponin and 5% BSA for 24h at 4ºC with anti-rhodopsin antibody (Rho4D2). Goat anti-mouse IgG AlexaFluor 488 was used as secondary antibody. The cell nuclei were observed with Hoechst. For images a microscope Operetta (Perkin Elmer) was used.

3.11.4. cAMP determination

The commercial assay CisBio BioAssay cAMp-GS Dynamic was used. It was based in specific antibodies marked with criptato (doner) and cAMP copled to d2 (acceptor). It is constitutated by several components: Stimulation Buffer 1 (5x), Lysis & Detection Buffer 2, IBMX, Forskolin, cAMP-d2 and anti-AMPc-cryptate. Foskolin was used as an activator of adenylate cyclase at 25 µmol/mL for 30 min to stimulate the cAMP pathway. The effect of several potential ligands of OR5P3 was evaluated: hexanol, carvone, cumarine and TLQP-62, by adding it to the cultured cells for 24 h. Tecan Infinite M1000 PRO was used to read the homogeneous time resolved fluorescence (HTRF) signal. The resulting data was analysed by GraphPad Prism 6, calculating the ration between the signal at 665 nm and 620 nm for each well. Results are expressed as the percentage of HTRF signal difference from the background measurements (%DF).

4 RESULTS

Here an effort was made to identify a possible receptor for VGF-derived antidepressant peptide TLQP-62. Receptors can be on the cell membrane or intracellularly, membrane bound or cytoplasmic. Although TLQP-62 is a 62 residues peptide and not expected to be able to cross or be transported across the cell membrane, total SH-SY5Y cell lysate was used to search for a possible receptor. As it is sill to unveil if TLQP-62 interacts directly with BDNF and is receptor TrkB or acts through other receptor, biotinylated TLQP-62 was crosslinked with SH-SY5Y cultured cells *in vivo* and its membrane fraction was further isolated and analyzed through affinity chromatography.

4.1 IDENTIFICATION OF HEAT SHOCK PROTEINS HSPA8 AND HSPD1 AND THE G-PROTEIN COUPLED OLFACTORY RECEPTOR OR5P3 AS TLQP-62 BINDING PROTEINS ON SH-SY5Y CELLS

For identification of a putative receptor or some binding partner of human TLQP-62 peptide, SH-SY5Y neuroblastoma cell line was used, as it is known to express high levels of VGF precursor.

4.1.1. Affinity purification of crosslinked biotin-TLQP-62 with SH-SY5Y membrane proteins

Biotinylated TLQP-62 was crosslinked with sulfo-EMCS and incubated with SH-SY5Y cells on plate for 2 hours at room temperature. After cells recovery, lysis and membrane fraction isolation, membrane lysate was analyzed through an avidin column. As control, no biotin-TLQP-62 was used, and only sulfo-EMCS was conjugated with cells and the resulting membrane lysate was passed through the column. Figure II.1 shows elution fractions, and several protein bands not visible in the control are seen in the reaction fraction (M). Bands (M1-M5) around 70, 60, 50, 45 and 25 kDa were analyzed through LC/MC for protein identification by Mascot (Table II.1).

Actin, tubulins, EEF1A1, PRDXs, and HSPA8 and HSPD1 were identified, which have been described as being present at cell membrane. Moreover, PCBP1, a cytosolic iron chaperone that passes iron to ferritin, and OR5P3, a G-protein coupled receptor (GPCR), were identified.

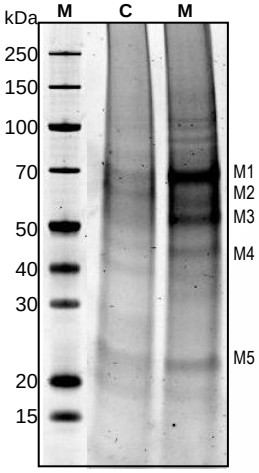


Figure II.1 | Affinity purification of biotin-TLQP-62-sulfo-EMCS crosslinked with binding proteins from SHSY5Y cell surface.

SDS-Page analysis in a 4-12% gradient gel followed by SYPRO ruby staining. **C** is the control sample containing SH-SY5Y membrane fraction crosslinked with PBS-sulfo-EMCS after avidn-affinity purification and **M** is the sample containing SH-SY5Y membrane fraction crosslinked with PBS-sulfo-EMCS-biotin-TLQP-62 after affinity purification. Protein bands **M1-M4** were excised and identified by LC/MS and MASCOTE.

TABLE II.1 | MASCOTE ANALYSIS OF PEPTIDES EXTRACTED FROM BANDS IN FIGURE IV.2

Band	Protein Identification	kDa	Nr peptides	Score
M1 (65-70 kDa)	Heat Sock cognate 71 kDa protein (HSPA8)	70,9	3	141,3
	60 kDa heat shock protein (HSPD1)	61,0	2	121,0
	Olfactory receptor 5P3 (OR5P3)	34,3	1	38,3
M2 (60 kDa)	Elongation factor 1 α (EEF1A1)	50,1	1	40,8
	OR5P3	34,3	1	33,8
M3 (55 kDa)	Tubulin β chain (TUBB)	49,6	2	99,5
	Tubulin α 3E chain (TUBA3E)	49,8	1	59,3
M4 (45 kDa)	Actin α (ACTA1)	42,0	4	183,7
	Poly(rC)-binding protein 1 (PCBP1)	37,5	1	45,1
	EEF1A1	50,1	1	39,7
M5 (~20 kDa)	Peroxiredoxin-1 (PRDX1)	22,1	6	265,9
	Peroxiredoxin-2 (PRDX2)	21,9	4	161,6
	Proteosome β 6 (PSMB6)	25,3	1	63,3

STRING tool was used to analyze the interactions between the identified proteins (table II.1 and figure II.1). HSPA8 is known to interact with all the other identified proteins, except PSMB6, VGF and OR5P3. In fact, these 3 last proteins are not known or predicted to interact with any of the other identified proteins (Figure II.2).

Considering all the identified proteins, and their described functions and cell localization, our attention was focused on OR5P3, and HSPD1 and HSPA8, since the first is a GPCR, and the last are chaperones that interact with each other, and can be found associated in lipid rafts in the plasma membrane to help maintaining its integrity and the structure of membrane proteins, such as receptors, including GPCRs[381]. Moreover, HSPA8 had been previously described as a binding partner, and putative receptor, of TLQP-21[380].

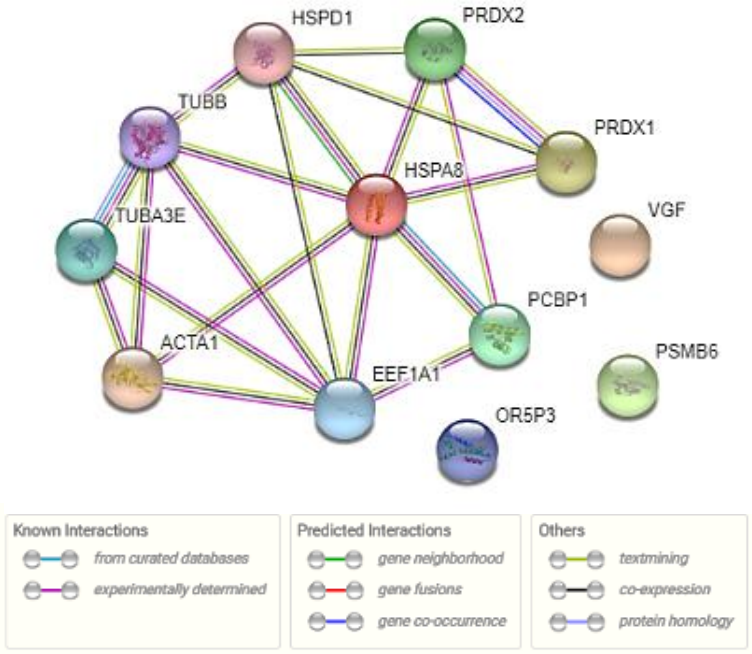


FIGURE II.2 | KNOWN AND PREDICTED INTERACTIONS BETWEEN THE RESULTING PURIFIED AND IDENTIFIED MEMBRANE PROTEINS FROM CROSSLINKING OF BIOTIN-TLQP-62 WITH SH-SY5Y CULTURED CELLS (TABLE II.1) USING THE STRING tool.

Olfactory receptors (ORs) were thought to be expressed only in the olfactory neurons present in the olfactory epithelium and to be responsible for the binding of volatile, water soluble or lipid soluble molecules that lead to the initial perception of smell in the brain. However, in the last years, more and more ORs were found to be expressed in non-chemosensory tissues and to be not directly related to the detection of odors[382]. ORs were found in kidney, lung, spleen, liver, pancreas, gastrointestinal tract, placenta, prostate, testis, erythroid cells and peripheral blood cells, eye, brain (*substancia nigra*, neocortex, hippocampus, dentate gyrus, striatum, thalamus, nuclei of the basal forebrain, hypothalamus, nuclei of the brainstem, cerebellar cortex, dentate nucleus and neurons of the spinal cord).

Expression of OR5P3 and HSPA8 on the SH-SY5Y cell line was confirmed by immunocytochemistry analysis. The resulting eluted fraction from the affinity purification of crosslinked biotin-TLQP-62 with SH-SY5Y membrane proteins was also analyzed by immuno-detection to confirm OR5P3 and HSPA8 presence.

4.1.2 Immunodetection of HSPA8 and OR5P3 as TLQP-62 binding proteins on resulting eluted from affinity purification of crosslinked biotin-TLQP-62 with SH-SY5Y membrane proteins

To confirm the identification of HSPA8 and OR5P3 as putative receptors or binding partners of human VGF-derived TLQP-62 peptide, immunodetection by western blotting and immunocyto-chemistry was performed. As primary antibody, rabbit IgG anti-human VGF(C-terminal), rabbit IgG anti-human HSPA8, rabbit IgG anti-human OR5P3 were used; and as secondary antibody, goat anti-rabbit IgG-HRP. Streptadivin-HRP was also used to confirm the presence of biotinylated protein complexes. Resulting bands are seen in figure II.3.

A double band corresponding to ~70 kDa is detected when using anti-HSPA8 IgG. HSPA8 has 70 kDa, but the presence of a double band around that molecular weight might indicate the complex *biotin-TLQP-62*sulfo-EMCS*HSPA8*. A higher molecular weight band of around 120-140 kDa is detected and might correspond to protein dimers or aggregates.

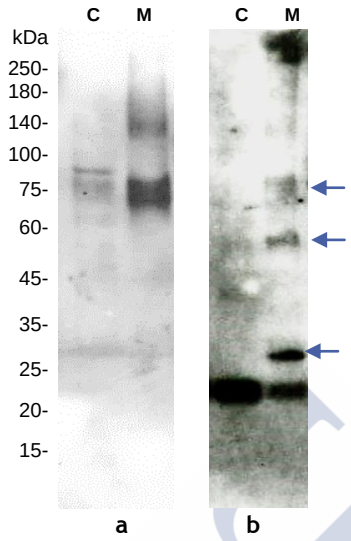


FIGURE II.3 | IMMUNODETECTION OF BIOTIN-TLQP-62 BINDING PROTEINS FROM SH-SY5Y CELL SURFACE. Western blotting analysis of resulting sample from affinity purification of biotin-TLQP-62-sulfo-EMCS crosslinked with proteins from SHSY5Y cell resulting membrane fraction. Membranes were probed with **a)** rabbit IgG anti-human HSPA8 and **b)** rabbit IbG anti-human OR5P3. As secondary antibody goat anti-rabbit IgG was used. **C** is the control sample containing SH-SY5Y membrane fraction crosslinked with PBS-sulfo-EMCS after affinity purification and **M** is the sample containing SH-SY5Y membrane fraction crosslinked with PBS-sulfo-EMCS-biotin-TLQP-62 after affinity purification.

Several bands were detected with anti-OR5P3 antibody: 70, 50, 25, and 20 kDa. The 20 kDa band was also detected in control and thus, might be an unspecific correspondence. OR5P3 has a molecular weight of 35 kDa, but GPCRs are usually found in dimers, or associated with cell membrane, and have post-translational modifications, like glycosylation, which alters its detected molecular weigh on SDS-PAGE analysis. ORs are seven-helix transmembrane proteins, with extracellular and intracellular loops, that bind to a G-protein. When solubilized from the membrane GPCRs can degrade, originating the separation of the seven-helix domains, and that will appear on the electrophoresis gel analysis. ORs were thought to be expressed only in the olfactory neurons present in the olfactory epithelium, but in the last years, more and more ORs were found to be expressed in non-chemosensory tissues, including the brain.

To confirm and make sure of the expression of OR5P3, and HSPA8, by SH-SY5Y cells an immunocytochemistry analysis was performed. As observed in figure II.4, SH-SY5Y cell line expresses VGF (that can be observed in vesicles close to dendrites/neurites), HSPA8 and OR5P3, being localized through whole cell, indicating its presence in the cytoplasm and most probably in the cell surface.

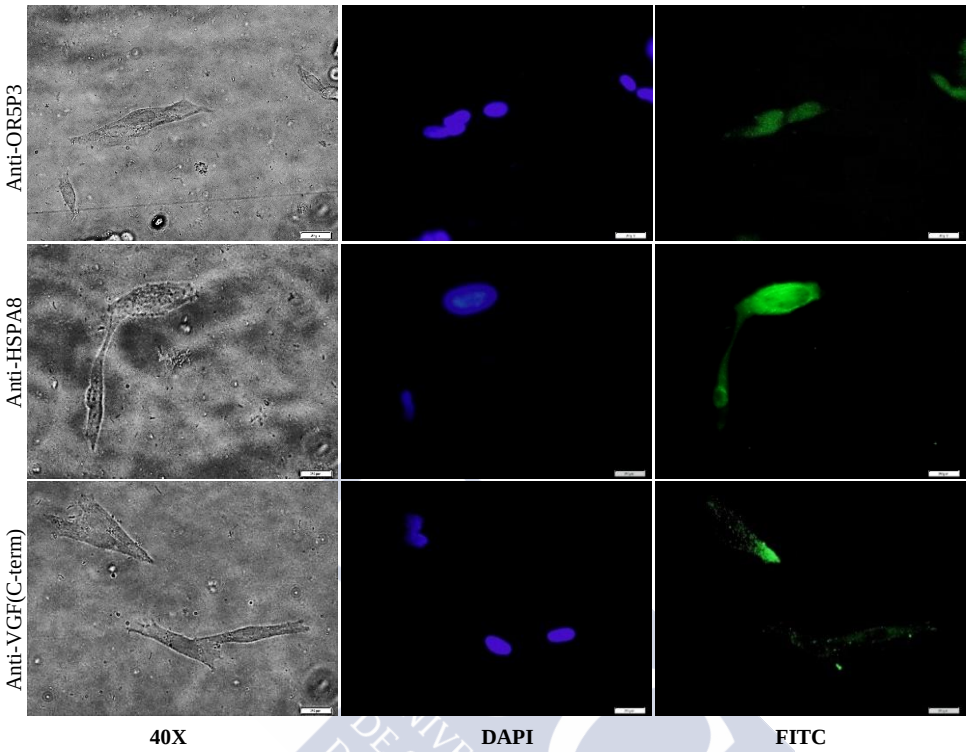


FIGURE II.4 | IMMUNOCYTOCHEMISTRY OF SH-SY5Y CELLS. As primary antibodies rabbit IgG anti-human VGF (C-term), rabbit IgG anti-human HSPA8 and rabbit IgG anti-human OR5P3 were used and goat anti-rabbit IgG-FITC as secondary antibody. DAPI was used to localize the nucleus in viable cells.

4.2 IDENTIFICATION OF TLQP-62 BINDING PROTEIN OR5P3 IN HUMAN HIPPOCAMPUS HOMOGENATE: AFFINITY PURIFICATION OF CROSSLINKED BIOTIN-TLQP-62 WITH HIPPOCAMPUS HOMOGENATE AND IMMUNODETECTION OF BIOTIN-TLQP-62 BINDING PROTEIN OR5P3

In an effort to confirm that the binding of these proteins to TLQP-62 is not an artefact, human hippocampus homogenate was used to perform crosslinking and affinity purification experiment followed by immunodetection analysis. After preparation of human hippocampus homogenate, fractions P1, S1, P2 and S2 (P for pellet and S for supernatant) were further isolated. Protein concentration was

determined before proceeding to the crosslinking experiment with biotin-TLQP-62. For detection of a putative receptor of human TLQP-62 peptide, hippocampus homogenate supernatant and pellet (membrane) fractions were used. Affinity purification using an avidin column to trap the biotinylated TLQP-62 crosslinked with sufo-EMCS was performed in order to trap and identify any protein able to interact and bind to TLQP-62. Elution fractions were precipitated in 85% cold methanol and ressuspended in Laemmeli buffer for SDS-PAGE analysis followed by SYPRO Ruby staining and compared to control fractions where no biotinylated peptide was used. The resulting gel and the immunodetection analysis performed on the same samples using rabbit anti-human OR5P3 is observed in figure II.5.

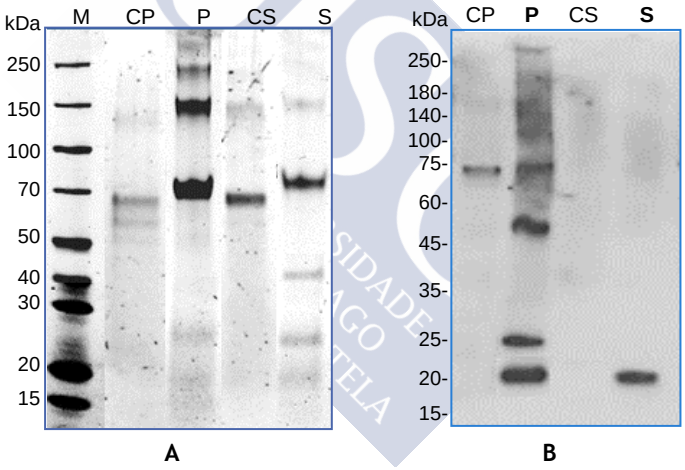


FIGURE II.5 | ANALYSIS OF BIOTIN-TLQP-62 BINDING PROTEINS FROM HIPPOCAMPUS HOMOGENATE. A) SDS-PAGE analysis and SYPRO ruby staining of resulting eluted from affinity purification of biotin-TLQP-62-sulfo-EMCS crosslinked with proteins from human hippocampus. **B)** Western blotting analysis with rabbit IgG anti-human OR5P3. As secondary antibody goat anti-rabbit IgG was used. P is for the resulting eluted fraction from hippocampus homogenate pellet fraction and S for the supernatant fraction crosslinked with PBS-sulfo-EMCS-biotin-TLQP-62 after affinity purification. As control sample containing hippocampus homogenate pellet (CP) and supernatant (CS) fraction crosslinked with PBS-sulfo-EMCS followed by affinity purification.

4.3 CHARACTERIZATION OF HSPA8-TLQP-62 INTERACTION

To evaluate the interaction between HSPA8 and TLQP-62 a dynamic mass redistribution analysis was performed using a range of different concentrations of peptide: 10, 20, 40, 60, 80 and 100 μM. For higher concentrations than 100 μM the peptide starts to precipitate and it is not possible to read the reflected wavelength shits properly. Response at 1 hour is shown in Figure II.6, indicating a weak interaction between the peptide and the chaperone, represented by a Kd of 155.2 μM.

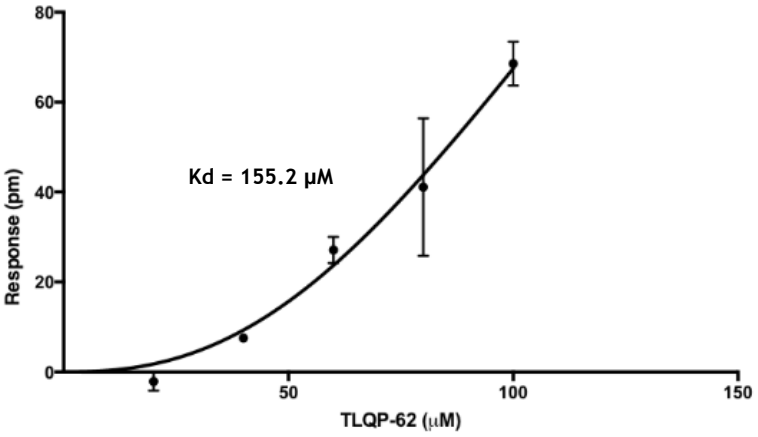
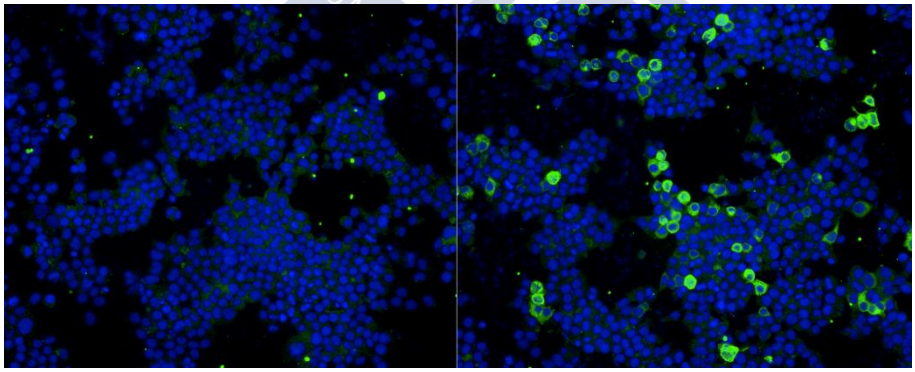


FIGURE II.6 | DYNAMIC MASS REDISTRIBUTION ANALYSIS OF TLQP-62 INTERACTION WITH HSPA8. A range of different concentration of peptide were tested, 10, 20, 40, 60, 80 and 100 μM. Response is shown at 60 min with a Kd of 155.2 uM. Label-free responses are measured as shifts in reflected wavelength and expressed in picometers (pm).

4.4 TLQP-62 IS A LIGAND FOR OR5P3

To prove TLQP-62 is an OR5P3 ligand, a cell model overexpressing this putative olfactory receptor was evaluate for this neuropeptide ability to increase cAMP levels. Hana3A cells were used to express the human OR5P3 by using a specific plasmid. As observed in Figure II.7, OR5P3 was successfully transfected into Hana3A cells and was being expressed on these cells.

To determinate cAMP levels, a commercial assay was performed. Cultured cells expressing OR5P3 were treated with several potential OR5P3 ligands – hexanol, coumarin and carvone – and TLQP-62 for 24h and cAMP levels were measured. As observed in figure II.8, an agonist activity was observed for TLQP-62 neuropeptide on the cAMP pathway in OR5P3 expressing cells, with an EC50 of 32.6 nM, and not in the control ones. None of the other odor ligands produces a similar effect, proving that OR5P3 function is most probably not connected to smell or odor.



Hana3A control *Hana3A pCI-OR5P3 transfected*

FIGURE II.7 | OLFACTORY RECEPTOR OR5P3 EXPRESSION IN TRANSFECTED HANA3A CELLS.
An immunocytochemistry assay was performed to confirm OR5P3 expression.

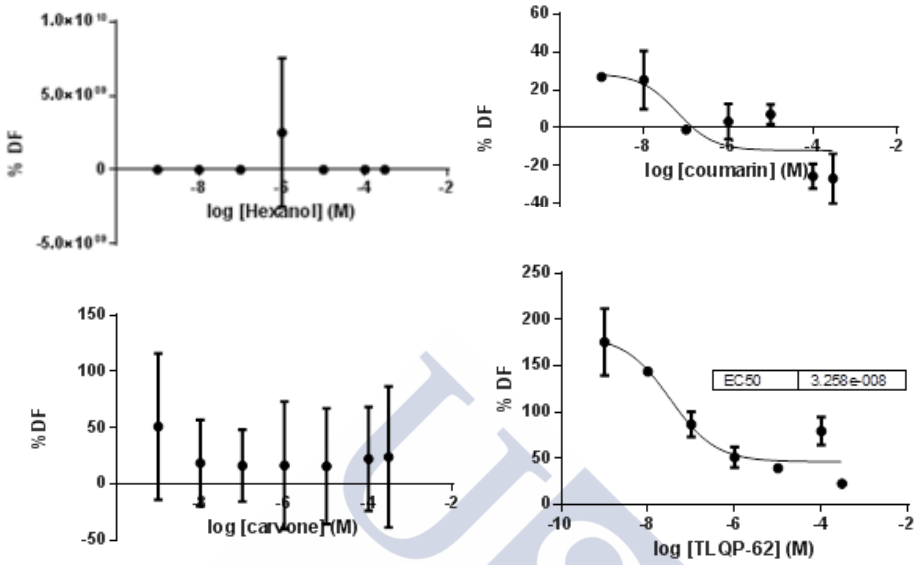


FIGURE II.8 | EVALUATION OF THE EFFECT OF SEVERAL POTENTIAL LIGANDS OF OR5P3 ON ITS COUPLED CAMP PATHWAY. Hexanol, coumarin and carvone were investigated as odorant ligand, to compare with TLQP-62. From all investigated ligands, only TLQP-62 succeeded by promoting a response via cAMP.

5 DISCUSSION

Considering the important role of human TLQP-62 antidepressant peptide on neuronal processes and neurological disorders, it is crucial the identification of a human receptor, not described to date, to better understand the underlying molecular mechanisms and signaling pathways, to pursue a treatment for several conditions, including depression and other neuropsychiatric disorders.

An effort was made to identify a putative human receptor for human TLQP-62, using SH-SY5Y cell line and human hippocampus homogenate as models of study. Membrane fraction of SH-SY5Y and hippocampus homogenate membrane fraction were used to perform a crosslinking experiment with biotin-TLQP-62. TLQP-62 was modified by attachment of a biotin molecule at the N-terminal via amide bound and with an extra cysteine residue to react with a crosslinker to facilitate the search for a receptor, and further purification by avidin affinity chromatography and detection by immunodetection using streptavidin-HRP. Biotin-TLQP-62 was first crosslinked with sulfo-EMCS, a heterobifunctional crosslinker containing a N-hydroxysuccimide ester, that reacts at pH 7-9 with primary amines by nucleophilic attack forming amide bonds, and a maleimide that reacts with sulfhydryl groups in cysteines at pH 6.5-7.5 forming stable thioester bonds[383,384]. Crosslinked biotinylated peptide was incubated with SH-SY5Y membrane fraction and purified by affinity chromatography for binding partners and possible receptor's isolation.

5.1 *OR5P3: TLQP-62 BINDING PARTNER AND PUTATIVE RECEPTOR*

As TLQP-62 has 62 residues and it is not expected to be able to cross or be transported across the cell membrane, there must be a receptor localized in the cell surface to transduce the signal. By crosslinking sulfo-EMSC-biotin-TLQP-62 with the SH-SY5Y membrane fraction and purifing any crosslinked complex using an avidin column, chaperones HSPA8 and HSPD1 were identified as

TLQP-62 binding partners, together with the G-protein coupled olfactory receptor 5P3 (OR5P3) (Figure II.1 and Table II.1). Several tubulins and actin were also identified, together with elongation factor 1A1 and peroxiredoxins. All of these proteins can be found in the cytosol, but also in the cell surface. Given the known functions and protein characteristics, heat shock proteins HSPA8 and HSPD1, and G-protein coupled receptor OR5P3 were selected to be further analyzed as putative receptors or binding partners of TLQP-62 with biological significance. Although OR5P3 was identified with a low score (<40), it was considered for further analysis, as the tissue used was only the cell membrane fraction.

OR5P3 is a G-protein coupled receptor, classified by homology as an olfactory like-receptor. Olfactory receptors (ORs) were thought to be expressed only in the olfactory neurons present in the olfactory epithelium, and that each olfactory neuron only expressed one type of OR[385]. However, more recently ORs were found to be expressed in non-chemosensory tissues, including the brain. Moreover, a single neuron outside the olfactory epithelium can express more than one receptor and the mechanism of transcriptional regulation may be different in olfactory epithelia and brain neurons[382,386,387]. ORs are described as being responsible for the binding of volatile, water soluble or lipid soluble molecules that lead to the initial perception of smell in the brain, and usually are co-expressed in the cells with one type of taste receptors. The signaling from the smell molecules from the olfactory epithelium goes to the olfactory bulb, then to the amygdala and then to the hippocampus, where memories linked to smell are produced by promoting hippocampal neurogenesis.

5.2 *CONFIRMATION OF TLQP-62 AND OR5P3 BINDING:*

To confirm OR5P3 expression on SH-SY5Y cells an immuno-cytochemistry analysis was performed on cultured cells using anti-human OR5P3 antibody where its presence was observed (Figure II.4). Also, OR5P3 immunodetection was performed in the eluted fraction resulting from the affinity purification of the crosslinked biotin-TLQP-62 with SH-SY5Y membrane fraction, as observed in figure II.3. Moreover, OR5P3 was also immunodetected in membrane fraction of human hippocampus homogenate (Figure II.5).

5.3 *CHARACTERIZATION OF OR5P3 AS A TLQP-62 RECEPTOR*

When a ligand bind to a OR it is activated and associated Golf proteins dissociate into subunits α, β and γ, and induce an increase of intracellular cAMP caused by the membrane form of adenylate cyclase 3 (AC3). To confirm TLQP-62 as a ligand of OR5P3 a cell model overexpressing this receptor was used: TLQP-62 was shown to increase cAMP levels through OR5P3 binding in the Hana3A cell model overexpressing this receptor. Several potential odorant ligands have been suggested to be OR5P3 ligands in the olfactory epithelium: hexane, coumarin and carvone. We evaluate for 24h the ability of these ligands to increase cAMP levels by activating OR5P3, and compared to the results when using TLQP-62 neuropeptide. Surprisingly, in Hana3A cells we could not detected an agonist activity for the 3 odorant ligands. Only for TLQP-62 was observed a cAMP activity indicating this neuropeptide is a ligand for OR5P3, with an EC50 of 32.6 nM. As none of the other odor ligands produces a similar effect, this proves that OR5P3 function is most probably not connected to smell or odor. Therefore, these preliminary results indicate TLQP-62 is a functional ligand of OR5P3 and its signal transduction in neurons is possibly linked to the expression of BDNF induced by TLQP-62, leading to neurogenesis.

5.4 *HSPA8 IS A TLQP-62 BINDING PARTNER*

On the other hand, we also found HSPA8 (HSC70), and probably HSPD1 (HSP60), to bind to TLQP-62. HSPA8 had been previously identified as a binding partner of TLQP-21[380]. Heat shock proteins are known for their chaperone functions, helping to proper folding proteins during cell stress, but also during normal cell functioning by helping to transport and fold nascent protein to its proper localization. HSPA8 and HSPD1 are constitutively expressed proteins that help in transport and folding of protein, inclusively membrane proteins, and can be found associated in lipid rafts in the plasma membrane helping to maintain its integrity and the structure of the membrane proteins, as receptors, including GPCRs[381,388,389]. HSPD1 prevents protein aggregation, and HSPA8 stabilizes and folds proteins. HSP60 are large oligomeric ring-shaped proteins known as chaperonins that bind partially folded intermediates, preventing their aggregation, and

facilitating their folding and assembly. These chaperones are found in all biological compartments except the ER. In addition to preventing aggregation, it has been suggested that HSP60 may permit misfolded structures to unfold and refold[390]. HSPA8, besides its chaperone function, is known to also carry out other important biological functions, as regulation of cell division, signaling, and transcriptional and translational control[391].

5.5 *CHARACTERIZATION OF TLQP-62 AND HSPA8 BINDING:*

To confirm HSPA8 expression on SH-SY5Y cells an immuno-cytochemistry analysis was performed on cultured cells using anti-human HSPA8 antibody where its presence can be observed (Figure II.4). Also, HSPA8 immunodetection was performed in the eluted fraction resulting from the affinity purification of the crosslinked biotin-TLQP-62 with SH-SY5Y membrane fraction (Figure II.3a).

Dynamic mass redistribution analysis was performed to evaluate the interaction between HSPA8 and TLQP-62, which indicated a weak interaction between the peptide and the chaperone, represented by a Kd of 155.2 μM, as observed in figure II.6.

5.6 *OLFACTORY RECEPTORS AND OR5P3*

More than 900 ORs genes and pseudogenes were identified from human genome sequences databases by homology[392]. The classification or nomenclature of OR genes is not fully established and its evolution is poorly understood. As illustrated in figure II.9, odorant signal transduction is initiated when odorants interact with specific ORs. ORs linked to Golf proteins are activated, by dissociating into subunits μ, β and γ, and induce an increase of intracellular cAMP caused by the membrane form of adenylate cyclase 3 (AC3). Increased intracellular cAMP causes an external Ca^{2+} influx by activating a cation-selective cyclic nucleotide-gated (CNG) channel. Then, rapid plasma membrane depolarization is triggered by the Ca^{2+}-activated Cl^- channel. The elevated intracellular Ca^{2+} concentration is reduced by expelling Ca^{2+} through the plasma membrane by a Na^+/Ca^{2+} exchanger (NCX), a potassium-dependent Na^+/Ca^{2+} exchanger (NCKX4), and plasma membrane Ca^{2+}-ATPase (PMCA). Olfactory marker protein (OMP) facilitates NCX activity and allows rapid Ca^{2+}

extrusion. The complex Ca^{2+}-calmodulin activates phosphodiesterase (PDE) that hydrolyzes cAMP and produces a negative feedback effect on the CNG channel[393–395]. Here, a CNGB3 channel was identified as binding to TLQP-62 on human hippocampus homogenate (data not shown). CNG channels are usually linked or very close to ORs in the plasma membrane, and as we also succeed to immunodetect OR5P3 in the human hippocampus homogenate as a potential TLQP-62 receptor, it is normal to also find CNGs associated.

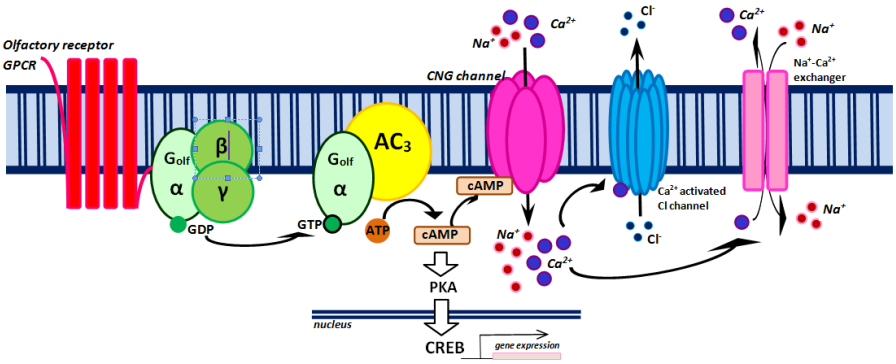


FIGURE II.9 | ODORANT SIGNAL TRANSDUCTION INITIATION.

[Constructed from data of Borisy *et al*. 1992, Pifferi et al. *2006* and Kang and Koo, 2012]

5.6.1. ORs in non-olfactory tissues

As mentioned before, some olfactory receptors are expressed not only in olfactory neurons but also in non-chemosensory tissues, where ORs are found to be co-expressed in the same cell type and also co-expressed with other GCPRs and taste receptors. In olfactory neurons, activation of the receptors elicits a receptor current, but activation of ectopically expressed receptors can have diverse effects. ORs are GPCRs, which can couple to different intracellular signaling cascades depending on the activation of different types of heterotrimeric G-proteins[396], the interaction with other cellular partners, such as arrestins and scaffolding proteins[397,398], hetero- or dimerization with other receptors and lipid-protein interactions[399,400]. Different G-protein subunits alpha ($G\alpha_S$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ or $G\alpha_{12/13}$) can trigger different signaling pathways, as illustrated in figure II.10. $G\alpha_S$ is stimulatory for AC3 leading to an increase in CAMP and $G\alpha_{i/o}$ inhibits AC3.

ORs are known to interact with a specific type of G-proteins, G_{olf}, similar to $G_{S,}$ but not much is known about signaling pathways mechanisms of ORs in non-olfactory tissues. GPCRs are activated by chemical ligands, as such small amines, peptide hormones, chemokines, lipids, proteins, ions, nucleotides and odorants, and can induce a variety of cellular responses, as cell shape changes and altered adhesion, cell migration, survival or proliferation[401,402].

ORs located in non-olfactory tissue are chemoreceptors triggering several responses not related to odor but rather to cell-cell communication and recognition, tissue injury, repair and regeneration, chemotaxis, cell division, growth and migration, nutrient sensing and regulation of blood pressure, energy homeostasis and cellular metabolism[387]. For example, ORs are expressed in pyramidal neurons in the cerebral cortex, suggesting its possible important role either in chemical detection of exogenous or endogenous ligands or in a developmental process, such as axon guidance and target recognition [386]. OR51E2 regulates cell proliferation and differentiation in human melanocytes and prostate epithelial cells[403,404]. Also, OR15 is expressed in pancreatic β-cells and promotes glucose-stimulated insulin secretion apparently not through G_{olf} but inducing PLC/IP3 downstream pathways, maybe suggesting $G\alpha_{q/11}$ involvement[405].

ORs are expressed in several tissues and organs, as the heart, brain, lungs, kidneys, placenta, liver, spleen, prostate, erythrocytes, eye and gut. Several molecules delivered from the blood, cerebrospinal fluid, neighboring local neurons and glial cells, distant cells through the extracellular space, and the cell self-regulating internal homeostasis, can be postulated as possible ligands for ORs. For example, ORs respond to short chain fatty acids produced by gut microbiota by regulating blood pressure[406], while activation of ORs by spicy species results in serotonin release[407]. Olfactory binding proteins (OBP) are small soluble extracellular proteins related to pheromone and odor transduction, mainly but not exclusively found in olfactory organs. OBPs are also present in several fluids, as vaginal discharge, urine, saliva, tears and amniotic fluid. OBPs in brain and other organs do not necessarily participate in olfaction, but rather are used in other metabolic functions which have nothing in common with smell.

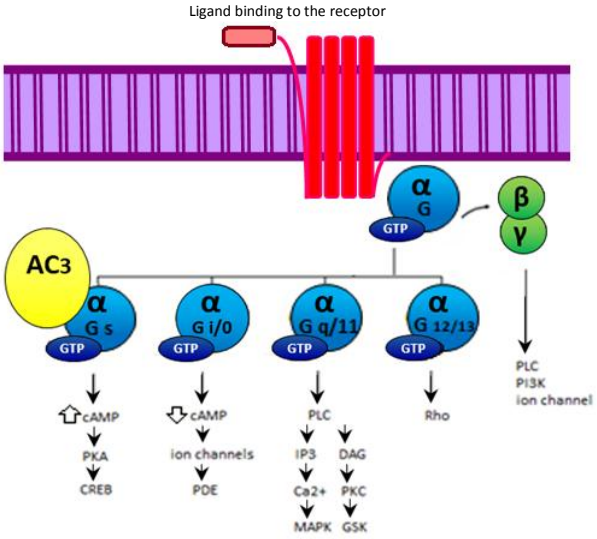


FIGURE II.10 | DIFFERENT SIGNALING PATHWAYS ARE ACTIVATED BY DIFFERENT G PROTEINS.
[Constructed from data of Luttrel, 2006]

5.6.2. ORs in non-olfactory organs of the nervous system

GPCRs are known to be essential in the regulation of structural plasticity and cognitive function by altering dendrite morphology and synapse formation through binding of neurotransmitters and neurotrophic factors. An example is brain-specific angiogenesis inhibitor 3 (BAI3), a p53 target gene encoding a adhesion GPCR expressed in central nervous system and crucial for synaptic plasticity in the hippocampus, and found to be altered in schizophrenia and other neuropsychiatric and neurodevelopmental disorders[142,408,409].

Several ORs have been found in dopaminergic neurons of *substantia nigra* and neurons of the spinal cord, in the cerebral and cerebellar cortex, hippocampus, dentate gyrus, striatum, thalamus and hypothalamus[382]. It is possible that neuropeptides can bind ORs in the brain, and other non-chemosensory tissues, to trigger homeostasis signals. Moreover, OR gene expression is known to be altered in several neurodegenerative diseases, including Parkinson's and Alzheimer's disease, prion disorders, and in depression and schizophrenia[185,186,382]. Also, taste receptors (TASRs) were observed in the mammalian brain, whose function is not related to taste[410–412].

TASRs in brain are also abnormally regulated in neurodegenerative diseases[413,414].

VGF and TLQP-62 are known to be expressed in olfactory bulb, hypothalamus, hippocampus, cortex, and cerebellum, and to have antidepressant properties and regulate memory by promoting neurogenesis in hippocampus. VGF-derived peptides are also known to have several functions in the glucose and energy homeostasis, response to insulin, bacterial defense, among others, and all these different functions might be accomplished through different olfactory receptors. TLQP-62 is known to act as an antidepressant regulating memory and learning by inducing neurogenesis, and thus, differentiation and dendritogenesis, by increasing BDNF/TrkB/PI3K/AKT/CREB pathway, which may be regulated through OR5P3. As described in the previous Chapter, TLQP-62 might even be responsible not only for TrkB phosphorylation but also its expression SH-SY5Y cells, and latter for BDNF expression. TrkB expression is known to be promoted via cAMP/CREB in neurons[315]. For further analysis TrkB levels should be investigated after SH-SY5Y treatment with TLQP-62.

5.7 *HEAT SHOCK PROTEINS: HSPA8 AND HSPD1*

In the CNS, HSPs have great importance not only on protein folding but also on processes such as synaptic transmission, stress response, protein kinase-mediated signaling as well as cell death[415]. HSPs have also been implicated in antigen presentation, immune stimulation, cell survival and cancer proliferation and neurological disorders, through signaling pathways as Ras/PKA or JAK/STAT3. Human and mice HSP70s have been show to localize at plasma membrane associated with lipid rafts and binding to specific lipids[416–418]. Moreover, it promotes traffic to the cell surface of several receptors including GPCRs, and mediates signal transduction[419]. HSPs are necessary for some ligands to bind their receptors, as is the case of glucocorticoid receptors that must be complexed with HSP90 and HSC70 to be activated and available to bind steroids[420]. Also estrogen receptor, progesterone receptor and androgen receptor ligand binding is controlled by HSP90[421–423].

HSP70 family is able to protect cells from proliferative and environmental stress factors due to its chaperone function. It plays key roles in cellular protein homeostasis by binding to exposed hydrophobic regions of incompletely folded or aggregated proteins. Also plays roles in immunity, transport, oxidative stress and signalling transduction. Current understanding of the role of HSP70 in protein folding suggests that the chaperone sequesters unfolded or partially folded protein, thereby preventing its aggregation, but does not actively participate in the folding process; subsequent binding of ATP leads to release of the substrate protein in a nonnative conformation. Both *in vivo* and *in vitro*, the transition of a protein from the unfolded to folded state frequently results in the formation of partially folded intermediate states that have a very strong propensity to aggregate, as illustrated in figure II.11[390]. *In vivo* this may lead to formation of inclusion bodies, especially when overexpression occurs. Members of the HSP60 and HSP70 molecular chaperone families seem to be most directly, and most generally, involved in preventing this.

HSPA8 is a constitutively expressed, cognate protein of the HSP70 molecular chaperone family, and enter the cell via endocytosis. It is an ATP binding protein with intrinsic ATPase activity which hydrolyzes ATP into ADP with conformational changes and substrate binding. HSPA8 binding to APT is enhanced by HSP40 and its dissociation by GrpE, forming a complex HSPA8/HSP40/GrpE (Figure II.12). HSPA8 has been shown to directly interact with NF-kB for nucleus translocation in hippocampal neurons. In the nucleus, HSPA8 regulates, in complex with CDK4 and

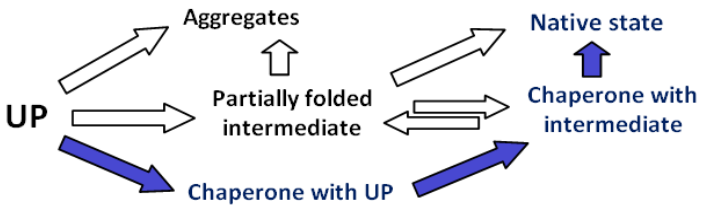


Figure II.11 | CHAPERONE-MEDIATED PROTEIN FOLDING IN CELL.
UP represents nascent unfolded polypeptide or newly synthesized protein. Chaperones include 40-, 60- and 70-kDa heat shock proteins.
[Constructed from data of Saibil, 2013]

p27, cell cycle progression. It has been found upregulated after activation of Wnt pathway and is associated with stem cell proliferation and apoptosis inhibition during development[424]. This chaperone is specifically localized in synapses, suggesting an involvement in synaptic signal transduction, and has been implicated in neurodevelopmental and neurodegenerative disorders[291,425]. Moreover it is induced by several factors, as heat and stress, but also, infection, injury, ethanol, estrogen, progesterone, drugs, glucose deprivation and physical exercise. Angiotensin II receptor (AT1aR) C-terminal interacts with HSPA8 for correct traffic and localization on the membrane. The same happens for the melanocortin-4 receptor[426].

HSPD1 is part of the HSP60 chaperone family, with large oligomeric ring-shaped proteins known as chaperonins, which facilitate protein folding by binding partially folded intermediates in their large central cavity, preventing their aggregation, and facilitating their folding and assembly. Folding can thus occur in a situation where aggregation is precluded. The HSP60 are found in all biological compartments except the ER. In addition to preventing aggregation, it has been suggested that HSP60 may permit misfolded structures to unfold and refold. Moreover, HSPD1 can specifically induce an inflammatory response and cell proliferation through several different receptors[427].

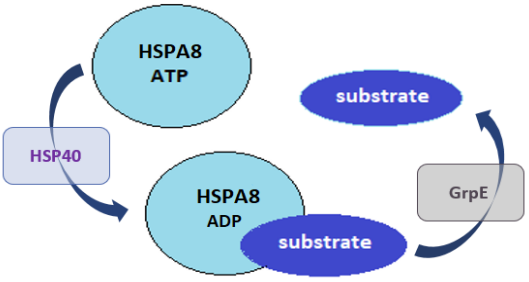


Figure II.12 | HSPA8 AND SUBSTRATE BINDING CYCLE.

In the ATP-bound state, HSPA8 has low affinity with the substrates. After hydrolysis of ATP with the ATPase activity, HSC70 in the ADP bound state binds with the substrates with high affinity. Some co-chaperones, as HSP40, enhance the ATPase activity of HSPA8 and GrpE enhance the dissociation of bound ADP from HSPA8 to allow the binding of ATP, resetting the cycle.

[Constructed from data of Saibi, 2013]

In the present study OR5P3, HSPA8 and HSD1 were co-purified with TLQP-62 indicating that all these proteins might be necessary to form a complex to allow TLQP-62 binding and OR5P3 activation for signal transduction. On the other hand, HSPA8 and HSPD1 might only be needed for proper folding and stabilization of TLQP-62 for further delivery to OR5P3 receptor and proper signal transduction.

Apparently TLQP-62 binds to OR5P3 causing a cAMP increasing which may suggest the involvement of, if not a G_{olf}, a G_S protein, stimulating AC3 increasing cAMP which might activate PKA/CREB pathway leading to the transcription of several genes, among them BDNF, which will lead to the activation of glutamate receptor and synaptic activity and neurogenesis. Further studies should be performed to unveil the cAMP-dependent pathway involved in this TLQP-62-OR5P3 interaction, and evaluate the levels of BDNF and other proteins involved in BDNF/CREB pathway.

Also, further studies concerning OR5P3, and HSPs, are to be performed in order to determine the nature of this interaction (if any) and unveil the downstream biological and molecular response of OR5P3-TLQP-62-HSPs interaction, but considering all the discussed data, these chaperones, OR5P3 and TLQP-62 possibly form a stable receptor complex, triggering a molecular cell response related to cell proliferation and differentiation in neurogenesis.

This could be of great importance for further investigation for an OR5P3 agonist to be used as an antidepressant treatment, based on TLQP-62 effects. Thus, in the next chapter the structure of TLQP-62 will be investigated by expressing it on *E.coli* for further purification and analysis of its secondary and tertiary structure by circular dichroism and nuclear magnetic resonance spectroscopy.

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CHAPTER III

Human TLQP-62

Structural Insights

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CHAPTER III

Human TLQP-62 Structural Insights

1 INTRODUCTION

VGF-derived peptide TLQP-62 is a small protein of 62 amino acids, with an important role in neurological processes. Proteins and peptides are responsible for carrying out fundamental biological molecular functions in the cell. Usually a protein is classified as a peptide when it has less than 50 residues. Because of its small size, peptides tend to be less well defined in structure and form random coils and so be more prone to aggregate, than proteins. However, peptides often have conformational preferences and adapt their native secondary structures, as helices and sheets. Peptides are regulators of the activity of other molecules, including proteins, and the function of a protein or peptide is determined by structure. Thus, knowing the three-dimensional structure of a peptide is of great help to understand how it will interact with other molecules, for example its receptor, and so its function. This facilitates the design of peptides or drugs (agonists/antagonists) with pharmaceutical use.

There are several methods to identify protein secondary and tertiary structure, including circular dichroism spectroscopy, X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy or electron microscopy. Also computer simulations, as molecular dynamics, are being used as an alternative tool to predict the structure of some peptides. Circular dichroism is used to unveil the secondary structure of a protein in solution. It is very important that the protein or peptide does not aggregate and conditions for best solubility and stability must be achieved. X-ray crystallography requires the protein to be in a crystal form and for that large amount of extremely pure

protein in solution is needed to test many different conditions for nucleation and growth of ordered crystals which will serve as a signal amplifier[428]. NMR also requires large amounts of isotopically labelled protein in solution but has slight lower resolution when compared to X-ray crystallography. NMR measures the quantum mechanical properties of the nuclei of atoms in proteins, determined by their local molecular environment, proving information on how close atoms are and how they could be linked chemically. These distance information can be used to determine protein tertiary structure[429].

As described in Chapter I, we investigated the effects of TLQP-62 neuropeptide in SH-SY5Y cells, which promotes its differentiation into a more neuron-like type cell, and its connection with DISC1. In Chapter II we identified OR5P3 as a receptor for this peptide in SH-SY5Y cells and human hippocampus homogenate. VGF has been implicated in some psycho- and neurological disorders, and described to be indirectly regulated by DISC1[148,187,191,193,430]. As TLQP-62 is known for having antidepressant properties - by promoting neurogenesis and having a role on memory and learning, through increasing BDNF expression – the identified receptor OR5P3 might be involved in this pathway and be a drug target for treating depression and other neurological disorders [150,190,431].

Thus, it is important to know more about the structure of TLQP-62 and OR5P3, alone and in complex, for further drug design.

2 OBJECTIVE

The aim of this chapter is to produce and purify TLQP-62 to further investigate its structural features by circular dichroism and NMR spectroscopy.

3 METHODS

3.1 HUMAN TLQP-62 PEPETIDE SYNTHESIS & PRIMARY SEQUENCE

TLQP-62 (human, molecular weight 7503 Da) was purchased from ChinaPeptides Co. LTd., Shangai, >95% pure, confirmed by HPLC and MS analysis, as a lyophilized powder. A stock solution of 1 mg/mL was performed in filtered PBS with 10% acetonitrile and stored at -80ºC. Peptide sequence is: **TLQPPSALRRRHYHHALPPS RHYPGREAQARRAQEEAEAEERRLQEQEELENYIEHVLLRR.**

3.2 SECONDARY STRUCTURE ESTIMATION

The secondary structure was predicted using the TLQP-62 primary sequence and the server ***Capito: a CD analysis & plotting tool*** (http://capito.nmr.leibniz-fli.de/) and the ***Swiss-Model tool*** from Expasy (http://swissmodel.expasy.org).

Circular dichroism spectroscopy experiment was performed in a J1500 circular dichroism spectrophotometer (JASCO) at 25ºC to evaluate TLQP-62 secondary structure. A solution of 0.1 mg/mL TLQP-62 in 50 mM NaH_2PO_4 pH 6, 7.5 or 9 was analyzed. The resulting data was analyzed also using ***Capito*** plotting tool.

3.3 TERTIARY STRUCTURE DETERMINATION: NMR

For human TLQP-62 expression the correspondent cDNA was cloned into a suitable vector for further transformation into *Escherichia coli* for expression. As hTLQP-62 is *a peptide of arou*nd 8 kDa, is susceptible to digestion by bacterial proteases and for better stabilization, solubility and longer half time life *in vivo*, a fusion protein was used to aid expres*ion and* solubility. The pETite™ N-6His SUMO vector (Figure V.1) allows the exp[re]ssion of the target protein as a fusion with an amino-terminal 6xHis-SUMO tag, which has been shown to increase the yield and enhance the solubility of a variety of proteins. SUMO (Small Ubiquitin-like Modifier) is a small protein (100 amino acids) recognized by the higly-specific SUMO

Express Protease, allowing precise removal of the tag to produce the target protein. The 6xHis motif at the amino terminus of the SUMO tag allows purification of the fusion protein by metal affinity chromatography. The vector is ready for co-transformation with the PCR product containing the gene of interest. The desired insert must be amplified with primers including 18 nucleotides of overlap with the ends of the vector. Recombination between the vector and insert occurs within the host strain, fusing the gene of interest to the vector.

3.3.1 Expression of isotopically labelled recombinant TLQP-62 peptide

pETite^{TM} N-6His SUMO vector: To clone human TLQP-62 cDNA into a vector for further expression on *E. coli*, the Expresso T7 SUMO cloning and Expression System (Lucigen) was used, containing pre-processed pETite^{TM} N-6His SUMO Kan vector, HI-control^{TM} 10G Chemically Competent Cells for cloning and HI-control^{TM} BL21 (DE3) Chemically Competent cells for protein expression and SUMO express protease for posterior SUMO cleavage.

Human TLQP-62 cDNA cloning: Human TLQP-62 cDNA was cloned as gene of interest into the pETite-N-6His-SUMO vector resulting in the recombinant vector pETiteN6HSUMO_hTLQP-62 expressing the recombinant protein 6HSUMOhTLQP-62 (Figure III.1). The hTLQP-62 cDNA sequence (Figure III.2) was inserted into pETite vector after amplification. A source of hTLQP-62 was required and for that purpose SH-SY5Y human neuroblastoma cell line was used.

M H H H H H H G S L Q D S E V N Q E A K P E V K P E V K P E T H I N L K V S D G S S E I F F K I K K T T P L R R L M E A F A K R Q G K E M D S L T F L Y D G I E I Q A D Q T P E D L D M E D N D I I E A H R E Q I G G T L Q P P S A L R R R H Y H H A L P P S R H Y P G R E A Q A R R A Q E E A E A E E R R L Q E Q E E L E N Y I E H V L L R R P Stop

FIGURE III.1 | 5'3' FRAME OF HTLQP-62 INTO PETITE N-HIS SUMO VECTOR.
In light blue, the histidine tag, in **black**, the sequence for SUMO and in underlined dark blue, the sequence for hTLQP-62.

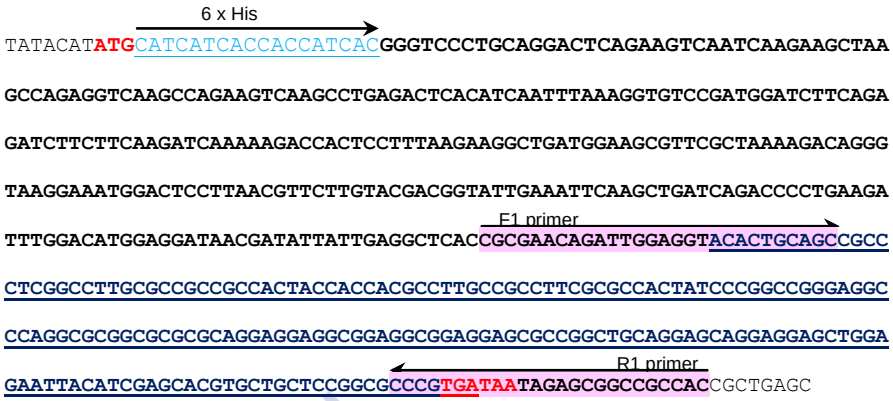

FIGURE III.2 | INSERTION OF HUMAN TLQP-62 CDNA INTO PETITE™N-HISSUMOKANVECTOR.
Vector has 2535 bp and hTLQP62 186 bp. In **light blue** is the sequence for the histidine tag, followed by SUMO sequence in **black**. The sequence for hTLQP-62 follows in **underlined dark blue**. Forward (F1) and reverse (R1) primer sequences used for cDNA amplification are indicated.

SH-SY5Y cell culture: The SH-SY5Y cell line (European Collection of Cell Cultures, 90430304) is a cloned subline of the neuroblastoma cell line SK-N-SH established in 1970 from a metastatic bone tumor of a four-year-old female (Biedler *et al*, 1973, 1978). Cells were maintained in a 1:1 proportion Earle's Balanced Salt Solution (Sigma-Aldrich) and F12HAM (Sigma-Aldrich) medium, supplemented with 15% Fetal Bovine Serum (Gibco, Life Technologies), 1% L-glutamine 200 mM (Gibco, Life Technologies), 1% MEM-Non Essential Amino Acids (Gibco, Life Technologies) and 1% penicillin-streptomycin (Gibco, Life Technologies) in 100x20 mm Falcon dishes (Life Sciences). Cells were grown at 37ºC in a 5% CO_2 humidified incubator. Confluent cell plates were washed twice with cold PBS and scrapped in 2 mL PBS. Cells were transferred to microtubes, centrifuged at 14000x*g* for 30 sec and supernatant discarded.

DNA extraction: genomic DNA extraction was performed using the RealPure Genomic DNA extraction kit (Real Laboratory) following manufacturer instructions. DNA was rehydrated by adding 50 µL of DNA hydration solution and incubating at 65 ºC for 1 hour with

shaking for DNA dispersion. DNA was quantified in a NanoDrop™ 2000 Spectrophotometer (Thermo Scientific) at 260 nm .

Polymerase Chain Reaction (PCR): hTLQP-62 cDNA contains 189 bp and to be inserted into the vector it must be amplified with primers including 18 nucleotides of overlap with the ends of the vector. The primer sequences F1 and R1 (Table III.1) were ordered to Sigma-Aldrich for further use in a PCR. The lyophilized primers were resuspended in H_2O_{MQ} to a concentration of 100 µM and 10 µM and stored at -20 ºC. Different DNA concentrations, temperature and time alignment were tested for PCR. A master mix reaction containing Takara DNA polymerase (Clontech, USA), 5x Takara buffer (Clontech, USA), dNPs (Clontech, USA), F1 and R1 primers (Sigma-Aldrich) were prepared with 3% DMSO in sterile H_2O_{MQ} (Table III.2). Purified genomic DNA (50 ng) was added. Control reactions were performed adding no DNA. PCR program reaction was performed in a thermocicler using a gradient for annealing temperature (Figure III.3). The PCR product (225 bp) was analyzed on a 1% agarose gel with SYBRO® Safe DNA Gel stain (Thermo Scientific), using the 1 kb DNA ladder marker (Genecraft Germany), quantified by absorbance at 260 nm and further cloned into pETite vector.

HI-control 10G cells transformation: Unpurified PCR product (50 ng) was mixed with pETiteNHisSUMO vector (25 ng) in a proportion 2:1, and left at room temperature for 5 minutes. For cloning and transformation, manufacturer instructions were followed. After transformation, cells were plated on LB with 30µg/mL kanamycin (LB^{Kan30}) and incubated at 37ºC, overnight. Several colonies were picked from the plate and ***a)*** used for colony-PCR, using the previously chosen conditions and same primers; and ***b)*** grown on LB^{Kan30} at 37 ºC, 225rpm, overnight, for later stock at -80 ºC in 15% glycerol, and for plasmid DNA extraction, using GeneJet Plasmid Miniprep kit (Thermo Scientific) according to manufacturer instructions. All centrifugations were performed at room temperature and 12000 rpm. Purified plasmid DNA was quantified by absorbance at 260 nm and stored at -20 ºC. After PCR, products were analyzed in 1% agarose gel with SYBRO® Safe DNA Gel stain and sent for sequencing.

TABLE III.1 | PRIMERS USED FOR PCR.
Forward and reverse primers sequences, length and alignment temperature.

Primer	Sequence 5' – 3'	Bp	Tm
Forward (F1)	CGC GAA CAG ATT GGA GGT ACA CTG CAG C	28	59
Reverse (R1)	GTG GCG GCC GCT CTA TTA TCA CGG G	25	59

TABLE III.2 | PCR MASTER MIX.
Volume and concentrations of reagents used.

Stock	[Final]
5x Takara buffer	1x
2.5 U/µL Takara polymerase	0.25 U
25 mM dNTPs	0.2 mM
10 µM F1 primer	0.2 µM
10 µM R1 primer	0.2 µM
DMSO	3%
DNA 370 µg/mL	0 / 50 ng
Sterile H_2O_{MQ}	-

FIGURE III.3 | PCR PROGRAM.
A temperature gradient for annealing was performed.

	Temperature	Time	
Denaturing	98 ºC	2 min	
Denaturing	98 ºC	30 sec	x40 cycles
Annealing	55 - 59 ºC	30 sec	
Elongation	72 ºC	30 sec	
Elongation	72 ºC	5 min	
Rest	4 ºC	∞	

HI-control BL21(DE3) cells transformation: Manufacturer instructions were followed for HI-control BL21(DE3) cells transformation with resulting pETite6HSUMOhTLQP62 vector. Transformed cells (100 µL) were plated on LB^{Kan30} and incubated overnight at 37 ºC. Colonies were picked and used for colony-PCR, and grown on LB with 30 µg/mL kanamycin for later stock and plasmid DNA extraction, as described before.

Isotope 6HSUMOhTLQP-62 expression: According to manufacturer instructions, protein expression was performed in LB^{Kan30} and 0.5% glucose at 37ºC, 225 rpm, overnight. HI-control BL21(DE3): :pETite6HSUMOhTLQP62 culture was inoculated in M9 minimal medium with 30µg/mL kanamycin and D-glucose-[$^{13}C_6$] (Cortecnet) and $N^{15}H_4Cl$ (Cortecnet) and kept shaking at 37ºC until optical density at 600 nm was 0.8-1. For induction, IPTG to a final concentration of 1 mM was added to the culture and continued shaking at 30 ºC, 175 rpm. Culture was harvest, centrifuged at 5000 rpm, 20 min 4ºC, resuspended in *lysis buffer* (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0, 1 mg/mL lysozyme, PMSF, DNase, β-mercaptoethanol) and incubated 30 min on ice with shaking. Cells were sonicated for 15 seconds (and 15 seconds interval) on ice, 5 pulses. Lysate was centrifuged at 12000xg for 30 min, and the supernatant containing the soluble protein was saved on ice for SDS-PAGE analysis and further protein purification.

Monodimensional (1D) SDS-PAGE: Protein extracts or eluted fractions were mixed with Laemmli sample buffer (Bio-rad) with 5% β-mercaptoethanol and boiled for 5 minutes at 99°C and loaded on 15% SDS-PAGE gels. All Blue Molecular Weight Marker (Bio-rad) and samples were applied on gel and subjected to electrophoresis in XCell Sure Lock system (Invitrogen) with electrophoresis buffer (25 mM Tris, 192 mM glycine, 1% (w/v) SDS, pH 8.3). The electrophoresis was performed for 10 min at 100 V followed by 50 min at 200 V and gel was stained with Coomassie solution for protein visualization and total protein was quantified.

Protein Quantification: Protein quantification was performed with Protein Reagent (Bio-rad). The standard calibration curve was prepared in duplicate for seven points of BSA in 20 µL of sample buffer at 1000, 800, 600, 400, 200, 100 and 50 µg/mL) and added to

780 µL of reagent. Blank was prepared by mixing 20 µL of sample buffer and 780 µL of reagent and for sample quantification, 2 µL of each sample was added to 800 µL of reagent in duplicate. Reaction was let to occur for 5-10 min and absorbance was measured at 595 nm in a Biomate 3 Spectro-photometer (Thermo Spectromic). Absorbance at 280 nm quantification was performed using a Nanometer 2000 (Thermo Scientific).

3.3.2 Purification of isotopycally labeled 6HSUMOhTLQP-62 recombinant protein

The strategy for protein purification is described in figure III.4

Immobilized Metal Affinity Chromatography: a HisTrap 5 mL nickel column (GE Healthcare) was cleaned with filtered and degassed ultrapure H_2O and equilibrated with 5 CV (column volume) ***Buffer A*** (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole, pH 7.6) before loading the cytosolic fraction (Sample A) at 2 mL/min using a Agilent HPLC system. Flowthrough was collected and column washed with 5 CV *Buffer A*. A gradient from 0-100% ***Buffer B*** (50 mM NaH_2PO_4, 300 mM NaCl, 750 mM Imidazole, pH 7.6) was applied at 1 mL/min for 40 min and 2 mL fractions were collected for further SDS-PAGE analysis. Selected fractions were pooled together and concentrated using a 15 mL 3 kDa cutoff Amicon (Millipore) concentrator (Sample B) and quantified.

Size Exclusion Chromatography: Sample B was dialyzed overnight against ***S200 Buffer*** (50 mM Tris-HCl, 100 mM NaCl, 5% glycerol, pH 8.0). A Sephadex 200 column (GE Healthcare) was cleaned with filtered and degased ultrapure H_2O, before equilibration with 2 CV *S200 Buffer*. Sample B was applied to the column at 0.5 mL/min for 1 CV (25 mL = 50 min) and 2 mL fractions were collected for further SDS-PAGE analysis. Fractions were selected, pooled together (Sample C), concentrated and quantified for further cleave of 6HSUMO using SUMO protease at 10 U / 10 mg protein, overnight at 4ºC (Sample D). Sample was analyzed by SDS-PAGE and purified by **S100** size exclusion using a as described before for TLQP-62 isolation and recovery. Resulting purified proteins were dialyzed overnight against 0.2 M ammonium acetate to be further lyophilized and kept at -80ºC.

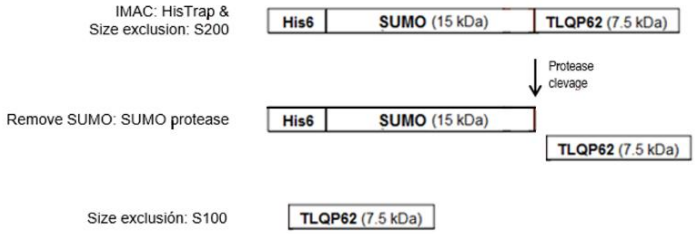

FIGURE III.4 | PROTEIN PURIFICATION STRATEGY.

3.3.3 Nuclear Magnetic Resonance Spectroscopy

Lyophilized 6HSUMO_TLQP-62, 6HSUMO and TLQP-62 proteins were dissolved in 20 mM $NaHPO_4$, 150 mM NaCl, pH 5.5, 5% D_2O to a concentration of 1 mg/mL and 0.6 mL were transferred to a 5 mm Shigemi tube.NMR analysis was performed at NMR Spectroscopy Unit at the *CACTUS* core facilities at University of Santiago de Compostela as decribed bellow:

NMR spectra were measured at 25ºC in a Varian Inova 750 spectrometer (750 MHz proton frequency) equipped with a HCN triple resonance probe with z-PFG capability and operating with standard 5 mm NMR tubes. The spectrometer operating software was TopSpin 4.0.x. NMR spectra were processed and represented with MestreNova 12.0 (Mestrelab Research Inc.).

One dimensional (1D) proton spectra (1D ^1H) were measured with the 1D water-control experiment for strong suppression of the H_2O solvent peak at ~4.7 ppm. The suppression of the solvent in this sequence was based in the watergate 3-9-19 scheme. The center of the spectrum was set at the solvent peak and the spectral width was 16 ppm. The relaxation delay (d1) and the FID acquisition ime (at) were 1.5 and 1 s, respectively. The spectrum was acquired with 512 scans in ~20 minutes.

The DOSY ^1H diffusion spectra were measured with the Double-Stimulate Echo sequence and Watergate solvent suppression (sequence Dbppste_wg of the Varian library). The spectrum was acquired with 9832 complex points in the ^1H dimension. The gradient pulses that encode diffusion were varied linearly between 2.66 and 53.3 G cm^{-}1 along 32 points in the diffusion dimension.

The duration of the bipolar gradients δ was 6 ms and the diffusion time Δ was 300 ms. The center of the spectrum in the ^{1}H dimension (F2) was set at 4.7 ppm and the spectral width was 16 ppm. The relaxation delay (d1) and the FID acquition time (at) were 1.4 and 1.5 s, respectively. The spectrum was acquired with 48 scans per each point in the diffusion dimension and the total measurement time was ~1h20min.

Two dimensional 2D HSQC ^{1}H-^{15}N spectra were measured with the sequence gNhsqc of the Varian library. The spectrum was acquired with 1706 and 128 complex points in the t2 and t1 dimensions, respectively. The INEPTs transfers of HSQC were optimized for a nominal value of 1JNH of 93 Hz. The relaxation delay (d1) and the FID acquition time (at) were 1.2 and 0.071 s, respectively. The center of the spectrum in the ^{1}H dimension (F2) was set at 4.7 ppm and the spectral width was 16 ppm. The center of the spectrum in the ^{15}N dimension (F1) was set at 120 ppm and the spectral width was 40 ppm.The spectrum was acquired with 128 scans per t1 increment. The total measurement time was ~11 h.

Two dimensional 2D HSQC ^{1}H-^{13}C spectra were measured with the sequence gCfhsqc of the Varian library. The spectrum was acquired with 2048 and 128 complex points in the t2 and t1 dimensions, respectively. The INEPTs transfers of HSQC were optimized for a nominal value of ^{1}JCH of 143 Hz. The elaxation delay (d1) and the FID acquition time (at) were 1.2 and 0.085 s, respectively. The center of the spectrum in the ^{1}H dimension (F2) was set at 4.7 ppm and the spectral width was 16 ppm. The center of the spectrum in the ^{15}N dimension (F1) was set at 80 ppm and the spectral width was 160 ppm. The spectrum was acquired with 16 scans per t1 increment and the total measurement time was ~1h30min.

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4 RESULTS

4.1 TLQP-62 SECONDARY STRUCTURE PREDICTION

For secondary structure prediction of human TLQP-62 the server ***Capito*** tool (http://capito.nmr.leibniz-fli.de/) was used. Based on the primary structure, the TLQP-62 peptide is predicted to form an α-helix in its C-terminal region and to form random coil in its N-terminal (Figure III.5A).

In agreement, the Swiss-Model tool, predicted an α-helix secondary structure for TLQP-62 based on the sequence and structure of Geminin, a nuclear protein of around 200 residues, with 30% identity with TLQP-62 (Figure III.5B).

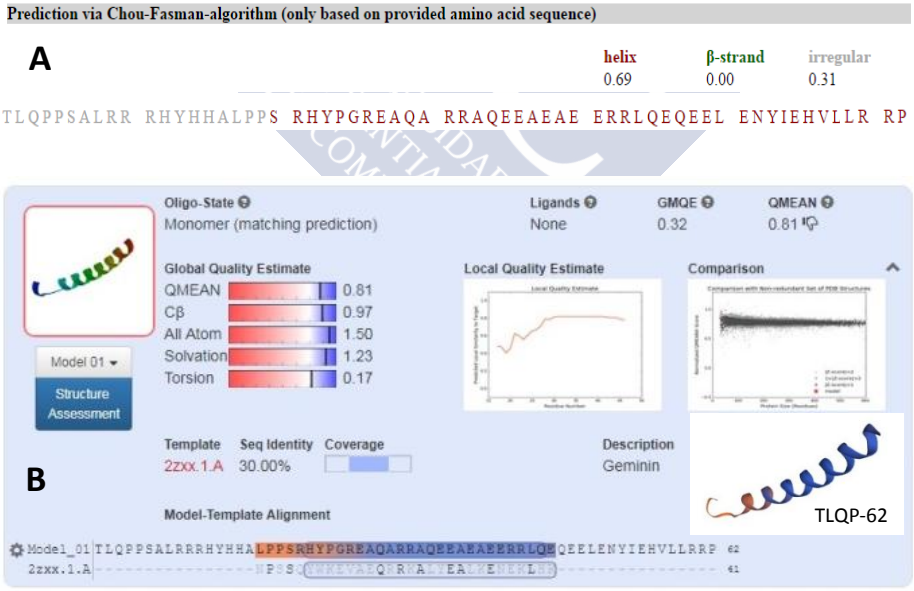

FIGURE III.5 | HUMAN TQLP-62 SECONDARY STRUCTURE PREDICTION. A) By Capito tool, and **B)** using Swiss-model tool with base on geminin sequence and structure

4.2 TLQP-62 SECONDARY STRUCTURE ESTIMATION

Data resulting from circular dichroism analysis of human TLQP-62 in solution at different pHs was analysed by *Capito* tool and shows no differences in the near-UV spectra (250-300 nm), but the far-UV spectra suggests this 62 residues neuropeptide behaves as a molten globule protein intermediate with a mainly disordered structure (random coil), that seems to be more stable at pH 6 and pH 9, compared to 7.5 (Figure III.6).

At a first sight, the resulting circular dichroism spectra seems to point to a protein rich in β-sheets, but in agreement with the predicted data could also indicate a disordered structure transitory with an α-helix, possible at its C-terminal, as a maximum absorbance peak is observed around 194 nm and a minimum absorbance peak at ~208 nm (Figure III.6). This data will be further discussed.

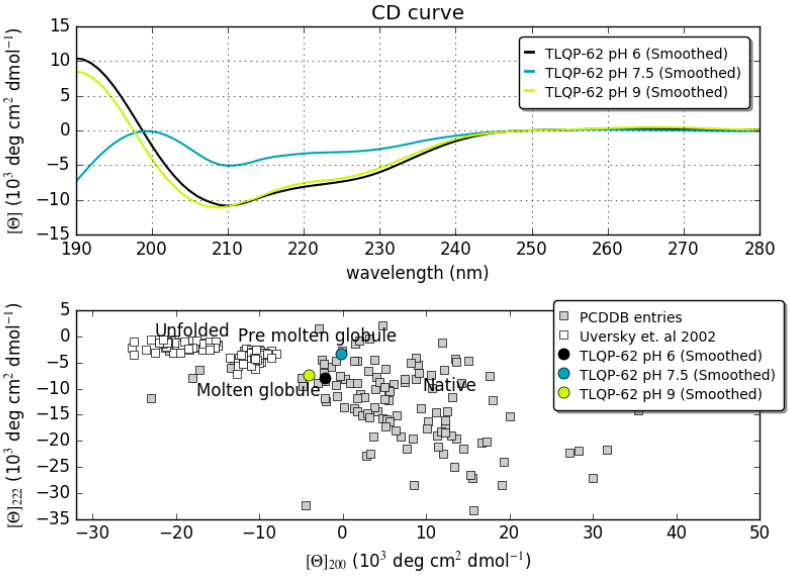

FIGURE III.6 | CIRCULAR DICHROISM ANALYSIS OF TLQP-62 AT DIFFERENT PHS BY CAPITO.
TLQP-62 behaves as a molten globule protein

4.3 PRODUCTION OF ISOTOPICALLY LABELLED 6HSUMOTLQP-62

NMR spectroscopy was used to further determine TLQP-62 three-dimensional structure. For that TLQP-62 must be produced with isotopes of ^{15}N and ^{13}C (and ^{1}H) for signal detection. As human TLQP-62 showed to have a mainly disordered structure it will tend to aggregate at high concentrations and to be cleaved by E. coli proteases when trying to be heterologous express it. Thus, a fusion protein, 6HisSUMO, was used to facilitate expression of a more soluble and ordered protein, for further purification and NMR evaluation.

4.3.1 6HSUMOhTLQP-62 expression

After bacterial growth of HI-control BL21(DE3) strain containing the recombinant vector pETite6HSUMOhTLQP62, recombinant protein expression was induced by adding 1 mM IPTG at 37ºC for different times. The resulting cell extracts were analyzed by SDS-PAGE to determine the best expression condition (Figure III.7a). As control no IPTG was aded to the bacterial culture. By its primary sequence 6HSUMO protein is predicted to have 17 kDa. Together with hTLQP-62 (7.2 kDa) its molecular weight is expected to be around 24 kDa. As seen is figure V.7 a ~25 kDa protein is present after 2 hours of induction with IPTG and same protein levels seem to be manteined until 16 hours of expression. A western blotting analysis was also performed using an anti-histidine antibody that recognize the 6xHis tag on the 6HSUMOhTLQP-62 protein (Figure III.7b).

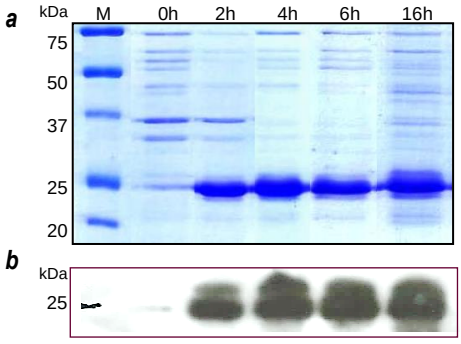

FIGURE III.7 | EXPRESSION OF 6HSUMOHTLQP-62.

a) A 12% SDS-PAGE analysis was performed on the resulting cell extract of HI-control BL21(DE3):: pETite6HSUMOhTLQP62 after induction with 1 mM IPTG for 0 to 16 hours. A ~25 kDa protein band is observed after 2 hours of induction.

b) A western blotting analysis was performed using a mouse anti-histidine primary antibody.

4.3.2 hTLQP-62 purification

The isotopically labelled recombinant protein was expressed at 37ºC for 6 hours for large scale production. Resulting cell culture was centrifuged, lysated, sonicated, centrifuged and ready for purification. The resulting samples analyzed by SDS-PAGE are shown in figure III.8.

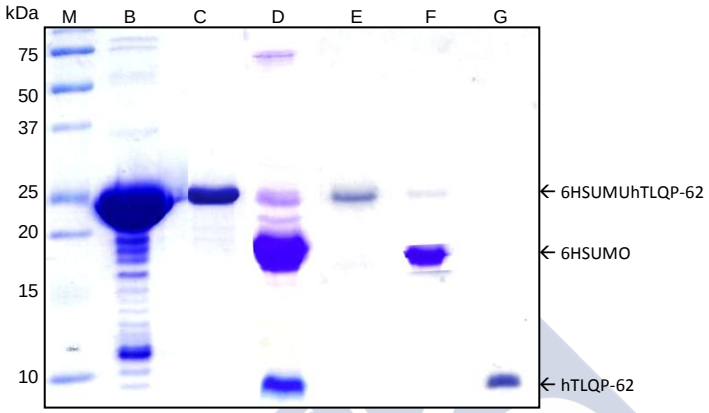

FIGURE III.8 | PURIFICATION OF 6HSUMOHTLQP-62. M, molecular marker; **B**, sample resulting from HisTrap affinity chromatography; **C**, Sample resulting from sample B size exclusion purification; **D**, sample C after cleaved with SUMO protease; **E**, **F** and **G**, corresponding to samples of 6HSUMOhTLQP-62, 6HSUMO and hTLQP-62, respectively.

Sample A was applied to a HisTrap column and the resulting flowthrought, wash fraction and elution fractions were analyzed by SDS-PAGE. As observed in figure III.8, the fraction containing a mainly pure 25 kDa protein, but still with some contaminants of lower molecular weight, (Sample B) was further purified by size exclusion, resulting in sample C. Sample C corresponds to pure 6HSUMOhTLQP-62 and was cleaved into 6HSUMO (18 kDa) and hTLQP-62 (7.5 kDa) by SUMO protease, originating sample D. Sample D was further purified by size exclusion into sample E, corresponding to 6HSUMOhTLQP-62 that was not digested, sample F, corresponding to 6HSUMO, and sample G, finally corresponding to hTLQP-62 isotopically labelled peptide.

4.4 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR)

A 2D ^{1}H-^{15}N HSQC experiment was first performed. In the HSQC one signal corresponds to a bonded N-H pair and is expected for each amino-acid, except for proline, which has no amide-hydrogen, and for asparagine, glutamine or tryptophan that appear as doublets on the top of the spectrum, because of the NH_2 from sidechains.

Spectra from well folded proteins have the peaks well-dispersed, and in which most of the individual peaks can be distinguished. In contrast, clusters of severely overlapped peaks in the middle region of the spectrum (7.5 to 8.5 ppm in the ^{1}H axis), indicate the presence of significant unstructured elements in the protein, with characteristic overlapping of the resonances of amino acid classes.

A HSQC for 6HSUMO (Figure III.9A) and 6HSUMOhTLQP-62 (Figure III.9B) were obtained and compared, since a hTLQP-62 HSQC of sufficient quality could not be obtained (data not shown) for apparently not enough pure peptide quantity or because the peptide is highly disordered and unstable.

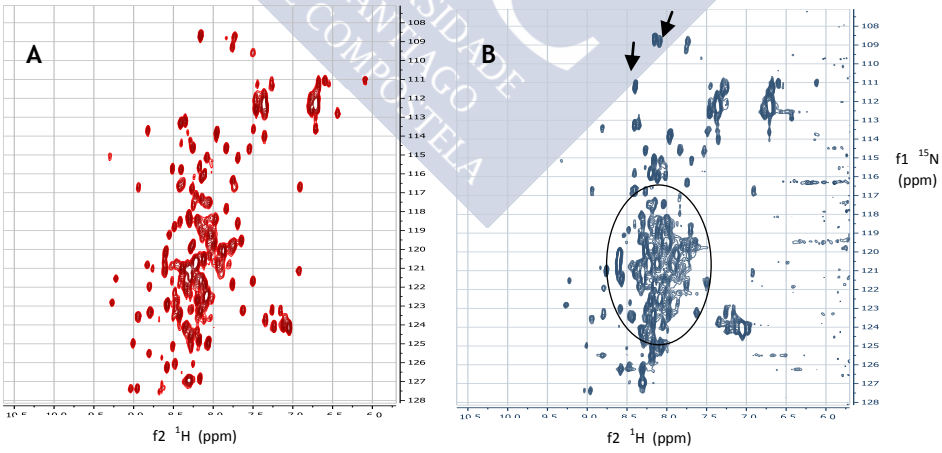

FIGURE III.9 | ^{1}H-^{15}N HSQC FOR A) 6HSUMO (RED) AND B) 6HSUMOHTLQP-62 (BLUE).
Arrows and black circle point to the spots seen in 6HSUMOhTLQP-62 and not in the 6HSUMO.

Human TLQP-62 samples were seen to be extremely labile, and degraded very quickly during manipulation (thawing, loading into NMR tubes and transfer to the spectrometer). After intense, yet futile, efforts to obtain a good spectrum from a hTLQP-62 sample, the alternative strategy of analyzing 6HSUMO and 6HSUMOhTLQP-62 was followed. As each signal corresponds to an amino acid it was reasoned that it would be possible to find the spots corresponding to hTLQP-62 peptides by subtraction of the spectra (Figure III.10).

However, apparently, even when bound to 6HSUMO highly soluble protein, most of the signals appear overlapped in the middle of the spectrum, as observed in figures III.9 and III.10, confirming that hTLQP-62 structure is mainly disordered in solution, even when bound to SUMO protein.

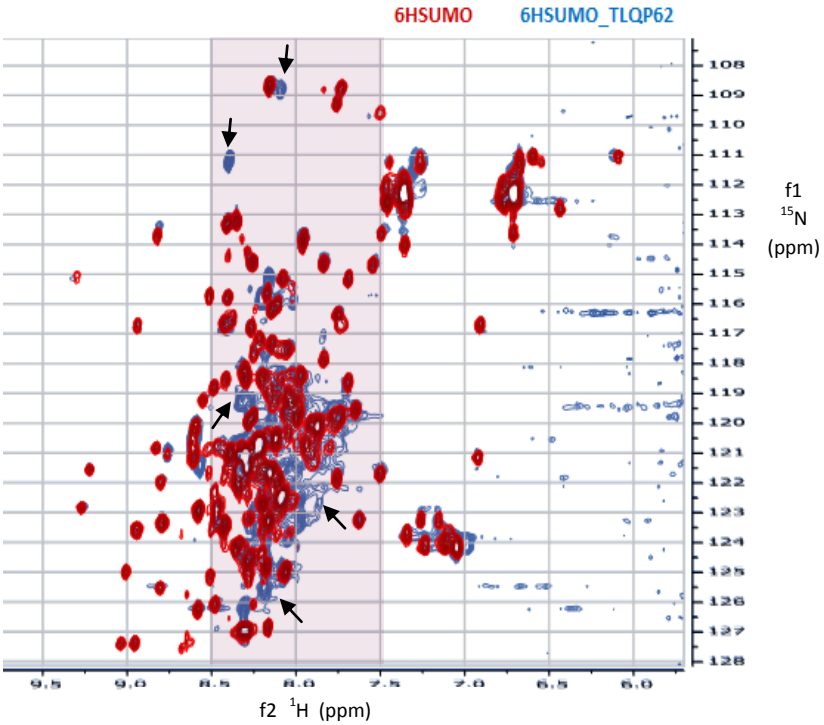

FIGURE III.10 | ^{1}H-^{15}N HSQC OVERLAPPING FOR 6HSUMOHTLQP-62 (red) AND 6HSUMO (blue).

5 DISCUSSION

Determining a peptide or protein three-dimensional structure helps to illustrate its potential use in academic, pharmaceutical or biotechnological industries. Knowing the structure of TLQP-62 can help to better understand its cellular and molecular functions and how it interacts with its receptor, OR5P3, identified in this present study.

5.1 TLQP-62: A RANDOM COIL TRANSITORY WITH A α-HELIX

Peptides are prone to be disordered once excised from their targets, due to its small size which causes a less well defined and stable structure. Circular dichroism (CD) spectroscopy is the unequal absorption of left- and right-handed circularly polarized light that can be used to determine protein secondary structure[432]. Far-ultraviolet CD (180-250 nm) corresponds to peptide bound absorption giving information about regular secondary structure (alpha-helix, beta-sheet, random coil). Near-UV CD reflects aromatic amino acid (tyrosine and tryptophan, mainly) side chains and gives information about conformational changes of a given protein in response to a given condition (ligand binding, T°C, pH, etc.)[432,433].

As observed in figure III.11A, a typical CD spectrum for an alpha-helix has a maximum positive absorption peak at 194 nm and minimum negative absorption peaks at 208 nm and 222 nm; for β-sheets a maximum positive peak at 195 nm and a minimum negative peak at 218 nm are observed; disordered proteins have a low ellipticity above 210 nm and a negative peak around 195 nm[432,433]. Although TLQP-62 is predicted to form an α-helix at its C-terminal region from its primary sequence, data from circular dichroism experiment shows this protein originates a spectra in a shape similar to that produced by β-sheet presence. However, a maximum absorption peak at 194 nm and a minimum absorption peak at 208 nm and another at 222 nm typical of α-helices are observed (Figure III.11B).

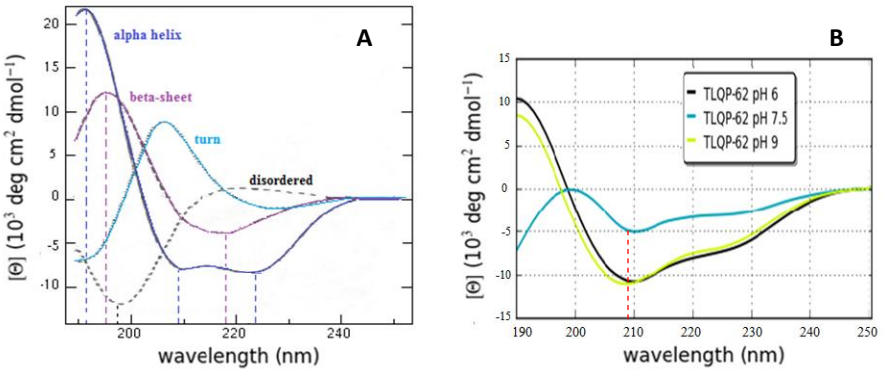

FIGURE III.11 | CIRCULAR DICHROISM ANALYSIS.

A) Example of spectra of proteins with alpha-helix, a beta-sheet, beta-turn or disordered secondary structure [Constructed from data of Greenfield, 2006, Bramhs & Bramhs, 1980 and Holzwarth & Doty, 1965]. **B)** Spectra resuting from TLQP-62 analysis indicating a combination of a disordered structure transitory with a α-helix that can be stabilized depending on the pH.

This may point to a cumulative spectrum indicating the presence of an alpha-helix but at the same time a random coil, behaving as a molten globule protein (Figure III.6). Apparenly at a pH near 7 its structure tends to be more disordered, which migh indicate that this peptide as a propency to be disorded in the cell.

Studies of protein folding make extensive use of CD to examine the folding pathway, as the technique has been especially important in characterising "molten globule" intermediates which may be involved in the folding process. Proteins could, at least, adopt 4 different conformations, ordered, molten globule, pre-molten globule and unfolded states[434]. Molten globule intermediates are a conformation assumed by many globular proteins under not optimum conditions and thus, with a very little thermodynamic stability, between the native and the fully unfold states[435]. Those proteins show the presence of substantial content of secondary structure, but absence of most of the specific tertiary structure produced by the tight pacing of residues side chain, indicating they might be a chaperone substrate for proper folding.

This may explain TLQP-62 binding to HSPA8 (and HSPD1) described in Chapter II, as partially folded intermediates tend to degrade or aggregate if do not bind to chaperones for proper folding. This finding could also explain why TLQP-62 degrades so quickly when trying to express it in *E.coli* or when in solution, once molten globule proteins are less folded and thus more accessible to proteolysis. Pre-molten globule proteins are even less compact than molten globule proteins and behave mainly as a random coil.

Accordingly, NMR resulting data also indicates that this small protein has a mainly disordered structure/random coil. Studies of NMR were performed in order to determine the tertiary structure of TLQP-62, however in solution the peptide alone showed to be highly unstable and to degrade and just few spots could be observed in the HSQC for 1H-^{15}N (data not shown) . In an attempt to stabilize the peptide, the fusion protein 6HSUMOhTLQP-62 was evaluated to check if it would be more stable, due to the presence of SUMO, a highly soluble protein that could help stabilize TLQP-62. When comparing the resulting pattern of 6HSUMO_hTLQP-62 with the one for 6HSUMO alone, one could infer that the expected extra ~60 spots in the 6HSUMO_hTLQP-62 would correspond to hTLQP-62 and its tertiary structure solved.

Indeed, more spots in the HSQC ^{15}N for 6HSUMO_hTLQP62 were observed than in the one for 6HSUMO, but the spots were fewer than 60 and pointed to a disorder peptide as mainly of the spots were in the middle of the spectra, which is characteristic of proteins and peptides that behave as molten globule proteins, since its unstable structure transiting between disorder-to-order causes to be difficult to read isotope signals. From TLQP-62 primary structure, this protein has 5 prolines, that would not appearing the HSQC spectrum, 1 glycine, that should appear on the top of the spectrum, and several aromatic residues that usually appears as doublets on the top of the spectrum.

This experimental study could be improved in order to better assess this peptide structure and confirm if this structure could be transitory with an α-helix, possible at its C-terminal. That helix might be stabilized upon binding to its receptor or other potential binding

partners, as HSPA8. To have better results it would be necessary to further stabilize this peptide. A study performed in the presence of SH-SY5Y was attempted but no results were obtained (data not shown). These cells express the TLQP-62 receptor OR5P3 and the chaperone HSPA8 that should bind to TLQP-62 and proper fold and stabilize it by inducing its α-helix conformation. Pure HSPA8 protein was also added to the peptide in solution, but again no signals could be obtained probably due to the size of HSPA8 that masks TLQP-62.

Although there is no doubt that TLQP-62 has a mainly disordered structure when in solution, further structural studies with the peptide and cells overexpressing OR5P3 should be performed to further investigate this complex structure and stabilization, which can be of great importance in determining a possible agonist to be used as an anti-depressant treatment through TLQP-62/OR5P3 pathway.

GENERAL DISCUSSION

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GENERAL DISCUSSION

Neuropsychiatric disorders affect a high percentage of individuals all over the world, having a great impact on society. These disorders are molecular and biologically poorly understood, which impacts on the efficacy of available treatments There are some evidence of some genes that are associated with schizophrenia, bipolar disorder and major depression, as is the case of DISC1, which was found to regulate VGF, a neuroprotein found downregulated in individuals suffering from depression and which expression can be rescued by antidepressant treatment and exercise[148,205,436] .

Neurotrophin-induced VGF is processed into several different bioactive peptides that are kept in vesicles and released from neuron cells in response to a stimulus. Those peptides have functions in glucose homeostasis, food intake or pain modulation, but also in neuroprotection, memory, learning, and cognitive function. Those processes are affected in patients suffering from neuropsychiatric disorders. VGF-derived neuropeptide TLQP-62 has an antidepressant-like effect in the hippocampus, apparently by promoting neurogenesis and enhancing BDNF levels. VGF is also induced by BDNF, suggesting there is a regulatory loop between these two proteins, which can even be regulated by DISC1.

TLQP-62 requires the TrkB receptor to exerts its effects, through BDNF and CREB, on proliferation and differentiation of hippocampal NPCs[150]. BDNF enhances glutamatergic neurotransmission and NMAD and mGluR5 receptors activation, further activating CaMKII and PDK, respectively, triggering a cascade modulating DNA synthesis and cell proliferation[150]. However, the specific molecular mechanisms by which TLQP-62 induces BDNF and exerts its effects, and its human receptor, are not known or described to date.

This interesting role of TLQP-62 in the brain, especially in the hippocampus, makes this neuropeptide an attractive target for further investigation of its role in neurogenesis, learning, memory and mental disorders, and possible treatment development.

Thus, the identification of the signalling pathways mediated by TLQP-62 (and its possible connection with DISC1) and of the TLQP-62 receptor(s), as well as both three-dimensional structures, is crucial for better understanding the molecular mechanisms of action of this neuropeptide for further investigation of agonists to be used as a treatment for chronic mental disorders.

In the present study, the effect of TLQP-62 on SH-SY5Y cells was investigated, as no morphological cell features had been described at the beginning of this study. TLQP-62 was found to have an effect on cell differentiation into a more neuron-like phenotype by enhancing neurite outgrow, more than in proliferation of these cells. In agreement, in a very recent study, TLQP-62 was also described as promoting dendritogenesis in hippocampal neurons[149]. TLQP-21 did not show any effect on differentiation, indicating that this effect is specific from TLQP-62 or of its C-terminal part. Moreover, VGF knockdown on SH-SY5Y cells causes them to be smaller, rounder and with a lower proliferative and survival rate over time. This indicates the important role of VGF in fundamental cell processes regulation. TLQP-62 was not capable of inducing differentiation on those cells, indicating a higher concentration of this peptide is needed or that another VGF-derived peptide(s) are essential for some process that allows TLQP-62 effect.

Retinoic-acid deficiency and abnormal TrkB signaling have been related to several neurological disorders. RA has also been described as inducing differentiation on this SH-SY5Y neuroblastoma cell line, promoting neurite outgrowth. This effect is mediated through enhancement of TrkB expression which leads to BDNF expression. Trk gene expression is induced by several compounds by increasing cAMP/pCREB on the cell[315]. SH-SY5Y cells are not responsive to TrkB before RA induction, and as far as known this differentiation effect occurs through TrkB and BDNF. As TLQP-62 is capable of inducing alone some neurodifferentiation degree on SH-SY5Y cells,

this might indicate this neuropeptide is capable of inducing TrkB expression. Neurite outgrow can also occur through p75 neurotrophin receptor, and probably NF-κB, which is expressed at basal levels in SH-SY5Y cells[314,437]. However, TrkB blockage studies show BDNF/TrkB/CREB pathway disruption, which is known to be necessary for TLQP-62-induced proliferation of hippocampal cells, and this effect is blocked[150]. Moreover, studies correlate VGF mRNA and TrkB mRNA localization in developing and adult rat nervous system[438]. Thus, p75 and TrkB expression and phosphorylation levels, must further be confirmed on TLQP-62-induced SH-SY5Y cells.

Neurotrophins regulate several crucial cell functions. Continued presence of the neurotrophins is required in the adult nervous system, for neuronal survival, morphology and differentiation. SH-SY5Y RA-induced differentiation can be supported by sequential treatment with BDNF alone[281]. The present study concluded that TLQP-62 is capable of supporting SH-SY5Y neurite outgrowth. Taking in account that VGF is a neurotrophin inducible protein that in turn is capable of induce BDNF, one might think that VGF, and its derived peptides, works as a regulatory protein of a BDNF-BDNF loop, to keep under control the necessary levels for each cellular process. A very recent study points out to a VGF(TLQP-62)/BDNF/TrkB regulatory loop in rapid antidepressant efficacy, together with mTOR pathway and AMPA receptor activation[439]. Other study suggests TLQP-62 proliferative effects requires NMDA and mGluR5, through CaMKII and PKD activation, respectively[150].

On the other hand, DISC1 also plays a role on cAMP signalling pathway, dopamine and glutamate signalling, synaptic activity, myelination, neuronal migration, interacting with several proteins related with the cytoskeleton, neuronal proliferation and differentiation, neurite outgrowth, and adult neurogenesis[174,184]. And, as shown in this study, both VGF and DISC1 knockdown in SH-SY5Y cells causes impaired RA-induced neurodifferentiation, with reduced neurite outgrowth, compared to wildtype cells. However, TLQP-62 sequential treatment is successful in supporting those cells continued differentiation.

As described, DISC1 knockdown results in a marked decreased of VGF expression, indicating that VGF expression is somehow regulated by DISC1, possibly through PI3K/AKT/CREB pathway[148,187]. It will be necessary to further evaluate DISC1 levels on VGF silenced cells, and check if there is a regulatory loop between VGF and DISC1 that can be responsible for those proteins function.

Based on the proteomic studies described here, TLQP-62 induces the expression of several proteins involved in several cell processes, on RA-differentiated SH-SY5Y cells. Proteins involved in cytoskeleton organization, axon growth, energy metabolism, protein biosynthesis, oxidative stress and immune response have their expression enhanced in the presence of TLQP-62, which explains the morphological chances and the ability for TLQP-62 to support neurite outgrow in the absence of RA. Some of those identified proteins have also been found dysregulated in hippocampus and cortices of individuals suffering from chronic mental disorders[124,127,289,291,440,441]. Neurotrophins regulate some of the processes mentioned above, including, axon growth, dendrite growth and the expression of proteins, such as ion channels, transmitter biosynthetic enzymes and neuropeptide transmitters that are essential for normal neuronal function. VGF is induced by neurotrophins and TLQP-62 is capable of inducing the expression of several proteins involved in those same processes, indicating that VGF is part of the neurotrophin-regulated pathways.

This study also describes the identification of OR5P3, a GPCR, as a TLQP-62 human receptor. The observed effects on SH-SY5Y cells morphology and proteossoma might happen through the activation of this receptor. As already discussed, OR5P3 is capable of enhancing cAMP levels. Cyclic AMP is a very important secondary messenger involved in several molecular pathways which activates protein kinases, as PKA, which in turn phosphorylate CREB leading to the transcription of several genes, as BDNF, VGF itself and TrkB expression. TrkB expression has been described as being regulated by cAMP/CREB pathway in neurons[315]. However, all of these supposed changes in those proteins expression are still to be confirmed by immunodetection in the SH-SY5Y cells.

On the other hand, TLQP-62 also binds to HSPA8, and apparently to HSPD1. TLQP-21 have also been described as binding to HSPA8[380]. HSP70 family has been shown to have no specificity for their client proteins, binding to several protein and having several roles intra- and extracellularly. HSPA8 specifically, besides protein folding, plays a role in protein transport and distribution, in signalling pathways and transduction, in immunity, autophagy and cellular stress. HSPA8 is induced by stress, heat, hypoxia, exercise, estrogen, progesterone, among others. It is present all over the cell, including bound to plasma membrane in lipid rafts[442]. It directs newly synthesized receptors to cell membrane, helping with its stabilization and, in some cases, with its activation, like is the case of glucocorticoid receptors[420]. Moreover, glucocorticoid receptors must be complexed with HSP90 and HSPA8 to be activated and available to bind steroids[420]. Interestingly, both HSPA8 and glucocorticoid receptors have been implicated in neurological disorders[10,381]. It has also been suggested that HSPA8 can act as a receptor for some virus cell entering during infection[443,444].

As TLQP-62 was found to be mostly disordered in solution, but forming a transitory α-helice in its C-terminal part, one could imagine that HSPA8 might participate on that proper folding stabilization for correct binding to OR5P3. HSPA8 and HSPD1 could, on the other hand, also be necessary to stabilize and activate OR5P3 and allow TLQP-62 binding, and thus forming a complex HSPs-OR5P3-TLQP-62. This complex would trigger a signalling pathway involving the increase of cAMP and probably PKA and the transcription factor CREB that will lead to the expression of several proteins, some of them probably identified in the 2D-DIGE proteomic study described in the Chapter II of this work, and also TrkB, BDNF and VGF, which in turn will active another genes involved in cell differentiation and neuritogenesis, wich, as described, could include genes involved in cytoskeleton organization, cell cycle regulation, oxidative stress, or immune response.

Figure GD.1 schematizes a global view of the findings described in this present work taking in account what has been described to date in the literature about TLQP-62 molecular mechanisms.

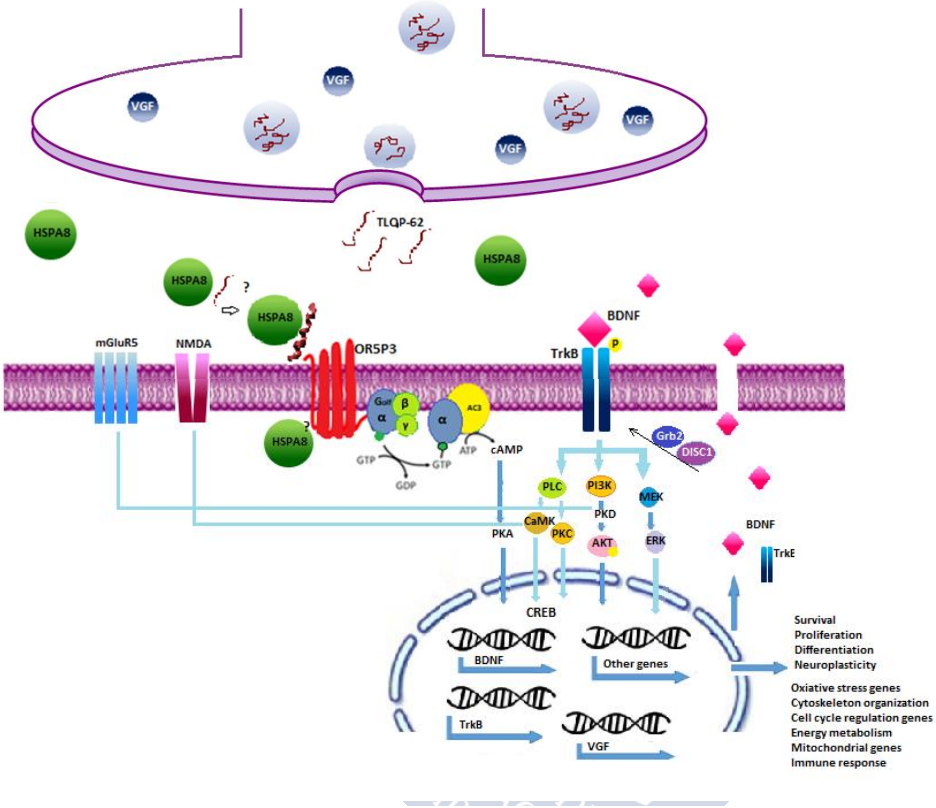

FIGURE GD.1 | POSSIBLE MOLECULAR MECHANISMS IN WHICH TLQP-62 MAY BE INVOLVED.

VGF is further processed in TLQP-62 and kept in vesicles in dendrites. Once TLQP-62 is released from presynaptic neuron it most probably has a disordered structure and need to be further folded and stabelized by HSPA8 (and most probably HSPD1 and other chaperones) to allow its binding to OR5P3 or even form a complex necessary for signal transduction iniciation. As shown in this study, TLQP-62 originates an increase in cAMP levels triggerimg a signaling pathway which leads to the expression of several genes. TLQP-62 has been observed in previous studies to increase VGF, BDNF and TrkB expression and phosphorylation levels, apparently through BDNF/TrkB/CREB pathway. At the sam time, BDNF as been described as potentiating mGluR5 and NMDA glutamate receptors activation and synaptic plasticity induction through PKD and CaMKII activation. All these signaling pathways lead to several genes expression involved in cell survival, proliferation or differentiation.

The present study contributes with new insights on VGF-derived peptide TLQP-62 molecular effects on neurdifferentiation, with a morphological and proteomic evaluation approach.

This peptide is capable of inducing neurite outgrowth, by arresting cell cycle and inhibiting proliferation, by inducing cytoskeletal and energy metabolism genes expression, and promoting cell survival through oxidative stress and mitochondrial genes regulation.

Understanding how this neuropeptide participates in synaptic and neurodevelopmental processes contributes to a better knowledge of its involvement in the pathophysiology of neuropsychiatric disorders and in the possible mechanisms of treatment.

Moreover, the finding of OR5P3 as a human receptor of this VGF-derived neuropeptide brings some enlightenment on its molecular mechanisms of action on those disorders and neurogenesis process.

Insights into TLQP-62 secondary and tertiary structure bring new knowledge into understanding its function and how it can possible interact and bind to its receptor, and other binding partners.

As further perspectives to better understand and characterize the potential impact of TLQP-62 on neurogenesis and neuropsychiatric disorders several studies should be further performed:

a. Studies concerning the OR5P3-TLQP-62 downstream signalling pathways to unveil the direct intermediates that lead to BDNF, and other genes, expression;
b. Structural studies of the OR5P3-TLQP-62 and HSPA8-TLQP-62 complexes should be performed and investigated for more insights into its structural features that may have pharmaceutical importance;
c. Search for an agonist for OR5P3 to mimic TLQP-62 effects should be pursued for pharmaceutical use as a putative treatment for some neuropsychiatric disorders.

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CONCLUSIONS

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CONCLUSIONS

1. Human TLQP-62 neuropeptide promotes SH-SY5Y neurodifferentiation with neurite outgrow.
2. Human TLQP-62 is capable of supporting SH-SY5Y RA-induced neurodifferentiation allowing cell survival in a BDNF-like way.
3. VGF knockdown causes TLQP-62 to fail promoting SH-SY5Y differentiation.
4. Proteomic study showed TLQP-62 induces several proteins expression in RA-differentiated SH-SY5Y cells involved in neurodevelopmental and synaptic processes.
5. Several of those proteins are involved in cytoskeleton formation and axon growth and guidance, in cell growth and communication, in energy and glucose metabolism and metabolites biosynthesis, and in oxidative stress and immune response.
6. Human TLQP-62 binds to OR5P3 G-protein coupled receptor in SH-SY5Y surface cells and in human hippocampus.
7. Human TLQP-62 induces an increasing in cAMP levels through OR5P3, suggesting this may be its human receptor.
8. Human TLQP-62 binds to HSPA8 in SH-SY5Y surface cells.
9. Human TLQP-62 is predicted to form a C-terminal α-helix from its primary sequence.
10. Human TLQP-62 in solution, from circular dichroism experiments, behaves as a molten globule protein, showing a disordered structure that may be transitory with a C-terminal α-helix.

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ANNEXES

RESUMO

As enfermidades neuropsiquiátricas afectan un 10% da poboación mundial tendo un grande impacto na sociedade. Porén, do punto de vista biolóxico e molecular non hai aínda moita información que permita un tratamento e diagnóstico efectivos, resultando nun elevado numero de suicidios por parte de pacientes mais graves.

As 5 principais enfermidades neuropsiquiátricas son o autismo, o défice de atención e hiperactividade, a depresión unipolar, a enfermidade bipolar e a esquizofrenia. A depresión é a principal enfermidade da sociedade moderna comprometendo a capacidade de interación e actividade social, podendo ser definida como a perda de interese polo mundo en xeral, acompañada de problemas de sono, apetito, enerxía, concentración, e sentimento de culpa, podendo estar asociada a síntomas de ansiedade. A enfermidade bipolar é caracterizada pola variación de síntomas entre a depresión e a euforia, con exceso de enerxía, actividade e autoestima, podendo ocasionalmente ocorrer síntomas psicóticos, como alucinacións, e síntomas de ansiedade e de defice de atención e hiperactividade. A esquizofrenia é unha enfermidade severa que causa grande incapacidade, podendo os síntomas ser positivos, con alucinacións e emocións, comportamento e pensamento desordenado; negativos, con apatía, catatonía ou anedónia; e cognitivos, con fraca memoria e concentración.

Todas estas enfermidades ten un compoñente xenético e ambiental, podendo ocorrer en membros de unha mesma familia, habendo algúns xenes suxeridos como candidatos a factores de risco ou biomarcadores. Os neurotransmisores ten un papel crucial na neuropatoloxia destas enfermidades e son a base do tratamento con antidepresivos, poren unha vez que non todos os pacientes responden ao tratamento, existirán outros mecanismos moleculares cruciais na regulación destas enfermidades.

Aínda que o diagnóstico sexa realizado por forma de intervista e observación do paciente polo médico, há evidencia de pacientes com o

hipocampo reducido, e numero, tamaño e densidade de neuronas diminuídos, indicando o envolvemento da función glutamatérxica asociada a memoria e función cognitiva diminuídas. Estudos proteómicos indican tamén alteracións en proteinas mitocondrias, citosqueléticas, sinápticas, metabólicas e imunolóxicas.

Estas alteracións son revertidas por antidepresivos, antipsicóticos ou exercicio físico, ao ter un impacto na neuroxénese. A neuroxénese é a xeración de novas neuronas a partir de células nai, ocorrendo principalmente no cérbero embrioxénico pero continua durante a vida adulta en células especificas do hipocampo e do bulbo olfactivo. Este proceso xera novas células que sofren migración, diferenciación con crecemento do axón e das dendritas, e formación de sinapses para poder integrar o complexo neuronal preexistente, conferindo plasticidade a estas rexións neuronais, regulando as emocións, aprendizaxe e memoria. Comprender os mecanismos moleculares asociados a neuroxénese adulta pode axudar a mellor comprender as enfermidades neuropsiquiatricas e a desenvolver mellores tratamentos. Unha vez que a neuroxénese está afectada nestas enfermidades, varios xenes, e as correspondentes proteínas, implicados en enfermidades mentais e neurolóxicas, e na neuroxénese adulta, ten sido albo de estudo, como BDNF (do ingles, *brain derived neurotrophic factor*), TrkB (*tyrosine kinase B*), CREB (c*AMP responsive element-binding protein 1*), DISC1 (*Disrupted in Schizophrenia 1*) e VGF (sen acronimo). Estas proteinas están implicadas na vía de sinalización BDNF/TrkB/CREB da neuroxénese hipocampal. DISC1 regula VGF vía PI3K/AKT/CREB na liña celular humana SH-SY5Y. VGF é inducida por NGF e BDNF, e é o precursor de varios outros pequenos neuropéptidos, como TLQP-62, que ten un efecto antidepresivo promovendo a neuroxénese hipocampal via BDNF/TrkB/CREB e aumentando a actividade sináptica e dendritoxénese, sendo un albo de investigación interesante na busca de tratamento para estas enfermidades.

DISC1 foi identificado nunha familia escocesa con varios membros afectados por varios tipos de enfermidades mentais asociadas a translocación (1;11)(q42.1;q14.3). Estudos xenéticos indican una relación entre DISC1 e memoria, cognición, volume reducido de masa cinza no cortex prefrontal e anomalías estruturais no hipocampo. Esta proteina é altamente expresa no hipocampo, cortex, bulbo olfactivo e cerebelo, e interactúa con varias outras proteinas relacionadas con o

crecemento de neurites, modulación do citoesqueleto, ciclo celular e transdución de sinal, como a tubulina, disbindina, FEZ1, Grb2, GSK3β, LIS1, MAP1A, NDEL1, PCM1 ou PDE4B/D. Alteracions na DISC1 orixina a disregulación da vía de sinalización de cAMP, da dopamina e do glutamato, e da neuroxénese.

Recentemente demostrouse que o silenciamento de DISC1 en celulas humanas de neuroblastoma SH-SY5Y, orixina a diminución de VGF, un xene de resposta a NGF (do ingles, *nerve growth factor*). DISC1 non interactúa directamente con VGF, pero regula a sua expresión a través da vía PI3K/AKT/CREB, ao interactuar e transportar correctamente Grb2 para dar inicio ao sinal de transdución. VGF é unha proteina precursora con un efecto antidepresivo ao promover a neuroxénese no hipocampo a través da vía BDNF/TrkB/CREB. Niveis reducidos de VGF foran descritos en pacientes con esquizofrenia, depresión, enfermidade bipolar, e outras enfermidades neurolóxicas.

A expresión de VGF é inducida por neurotrofinas, como neurotrofina-3 ou BDNF, e pola serotonina e fármacos que aumenten os niveis deste neurotransmisor. A proteina resultante é expresa en neuronas no cerebro, espinal medula e órganos neuroendócrinos, e regulada en resposta a diferentes estímulos que orixinan diferentes pequenos neuropéptidos que son almacenados en vesículas secretorias nas dendritas. Estes neuropéptidos están envolvidos na regulación da homeostase de enerxía, da glucosa e da auga, e do metabolismo, na función gastrointestinal, na reprodución, na modulación da dor, e na memoria e aprendizaxe, regulando a plasticidade sináptica e neuroxénese.

Os peptidos TPGH, NERP-1, NERP-2, NERP-3, NERP-4, NAPP-129, TLQP-62, TLQP-21, TLQP-24, AQEE-30, AQEE-11, LQEQ-19 deviran da VGF e son bioactivos. NERP-1, -2 e -3 regulan a homeostase da auga, NERP-2 regula a inxestión de comida, a secreción gástrica, a temperatura corporal e consumo de oxixeno. TLQP-21 previnde a obesidade, estimula as vías catabólicas, ten propiedades analxésicas modulando a dor, diminui a presión sanguina e reduce a apoptose neuronal *in vitro*. TLQP-62 e AQEE-30 regulan a función sináptica, inducen a neuroxénese e ten un efecto antidepresivo. VGF foi proposto como biomarcador para a esclerose lateral amiotrófica, enfermidades de Parkinson e de Alzheimer, e

demencia frontotemporal. Está tamén proposto para a esquizofrenia, depresión e enfermidade bipolar. Administración de TLQP-62 ou AQEE-30 en celulas hipocampais produce un aumento da actividade sináptica e da vía BDNF/TrkB/CREB, orixinando un aumento na proliferación neuronal e un efecto antidepresivo. Os receptores do glutamato mGluR5 e NMDA parecen mediar o efecto antidepresivo de VGF e TLQP-62, inducindo a proliferación celular e a actividade sináptica. A proteina NPAS3 (*neuronal PAS domain protein 3*), un factor de transcrición asociado a enfermidades neuropsiquiatricas, induce a expresión de VGF a través da vía NfkB (nuclear factor kB), aumentando a proliferación neuronal. Asín, 3 proteinas asociadas a enfermidades neuropsiquiátricas, DISC1, BDNF1 e NPAS3, regulan a expresión de VGF, facendo desta proteina e dos seus péptidos un albo farmacéutico interesante para o desenvolvemento de novas terapéuticas e fármacos para o tratamento estas enfermidades. Unha vez que os péptidos C-terminais, TLQP-62 e AQEE-30 parecen ser os responsábeis polo efecto antidepresivo, son os albos de estudo mais atractivos.

TLQP-62 é un neuropéptido composto por 62 residuos, do cal AQEE-30 faz parte da sua rexión C-terminal. Ten funcións na modulación da dor, modulación da homeostase da glucosa e da secreción de insulina, na memoria e aprendizaxe, na plasticidade sináptica e na neuroxénese hipocampal, a través da via BDNF/TrkB/CREB. Este péptido é un albo de investigación moito atractivo para mellor comprender os seus mecanismos moleculares, a sua ligazón con DISC1, e o seu envolvemento nas enfermidades neuronais. Non hai, ata o presente, ningún receptor identificado para este neuropéptido, nen vía de sinalización asociada. Esta identificación poderá permitir o desenvolvemento de agonistas como tratamento desas enfermidades.

No presente estudo, dividido en tres capítulos, avaliaranse os efectos de TLQP-62 humano na diferenciación da liña celular SH-SY5Y, tentouse encontrar un receptor para TLQP-62 e aínda, determinouse a estrutura tridimensional deste péptido.

Para mellor comprender o efecto de TLQP-62 humano na neurodiferenciación e proliferación celular, foi analizado o seu efecto en células humanas de neuroblastoma SH-SY5Y, asín como a sua ligazón a DISC1, por avaliación de alteracións morfolóxicas e

proteomicas. VGF e TLQP-62 son altamente expresos no hipocampo e inducidos por BDNF. Por sua vez TLQP-62 promove a indución de BDNF e a activación do seu receptor TrkB, orixinando un *loop* regulador controlando a proliferación celular e actividade sináptica, pero no está claro como e que vías son usadas para que VGF e BDNF promovan a expresión un do outro. BDNF interactúa con o seu receptor TrkB e pode activar tres vías diferentes: PLCy, implicada na plasticidade sináptica; PI3K, implicada no crecemento celular; e ERK, envolvida na diferenciación neuronal e crecemento de neurites.

Como descrito, a DISC1 está tamén implicada na neuroxénese, no desenvolvemento neuronal e plasticidade sináptica, e indirectamente regula a expresión de VGF vía PI33K/AKT/CREB. Investigar e comprender mais sobre os papeis da DISC1 e VGF na neuroxénese e neuritoxénese pode axudar a desenvolver novas formas de tratamento para as enfermidades neuronais.

Cultivos primarios de rato son usadas no estudo de varios aspectos neurolóxicos, pero son necesarios celulas humanas para validar posibles mecanismos moleculares de enfermidades humanas. A liña celular humana SH-SY5Y reproduce propiedades bioquímicas e morfolóxicas de neuronas, sendo usada como modelo *in vitro* de neuronas humanos. Esta liña celular pode ser diferenciada en celulas con un fenotipo mais neuronal, exhibindo neuritas, e expresa elevados niveis de VGF. Eses niveis de VGF sofren diminución en celulas silenciadas para DISC1. O ácido retinoico induce a diferenciación das celulas SH-SY5Y con crecemento de neuritas, ao inducir a expresión de TrkB, e activando as vias BDNF/TrkB/CREB. Como TLQP-62 induce BDNF, foi avaliado o efeito deste neuropéptido na proliferación, diferenciación e proteoma de celulas SH-SY5Y.

No presente estudo trataranse celulas SH-SY5Y con TLQP-62 e verificouse que este neuropéptido ten a capacidade para inducir a diferenciación destas células promovendo o aumento das neuritas. TLQP-62 ten a capacidade de soportar a diferenciación de celulas SH-SY5Y diferenciadas con ácido retinoico e orixinar celulas completamente diferenciadas con dendritas que ligan entre si.

Celulas con VGF silenciado son mais redondas ten una taxa de proliferación e de sobrevivencia mais baixa, e unha resposta à diferenciacion promovida pelo ácido retinoico mais lenta, podendo indicar que VGF é crucial para o desenvolvemento neuronal.

Deficiencia de ácido retinoico e anomalías de expresión de TrkB están envolvidas en enfermidades neuronais, estando asociadas a neuroxénese hipocampal e memoria, xunto con as neurotrofinas. O ácido retinoico induce a expresión de receptores Trk, o que permite que as celulas SH-SY5Y respondan a neurotrofinas, como BDNF, NGF, NT-3 e NT-475, que regulan o crecemento de axón ou dendritas, e a expresión de proteinas, como canais iónicos, encimas necesarias à biosíntese de neurotransmisores, ou neuropéptidos envolvidos na neurotransmisión, como VGF. Asín, o BDNF soporta e promove a neuritoxénese e sinatoxénese en celulas tratadas con ácido retinoico. No presente estudo, demostrouse que TLQP-62 é capaz de inducir a neuritoxénese en celulas SH-SY5Y e de soportar a diferenciación promovida polo ácido retinoico, na ausencia deste. Estes dados suxiren que TLQP-62 promove a expresión de BDNF pero tamén de TrkB. Outros estudos demostrarán que o bloqueo de TrkB case elimina por completo os efectos antidepresivos inducidos por TLQP-62.

Células silenciadas para DISC1 exhiben un défice no crecemento de neuritas, en numero e tamaño, na presencia de ácido retinoico, comparado con SH-SY5Y *wildtype*. Celulas silenciadas para DISC1 e células silenciadas para VGF, foran crecidas durante 9 días en presencia de ácido retinoico, mais 3 días con TLQP-62. En comparación con celulas *wildtype* e celulas a sobreexpresar DISC1 con o mesmo tratamento, as células silenciadas exhiben menos neuritas e mais curtas, evidenciando que VGF e DISC1 ten un papel fundamental na neurodiferenciación e neuritoxénese. DISC1 pode ser importante para manter regulados os niveis de VGF e de BDNF durante a proliferación ou diferenciación celular.

Celulas SH-SY5Y foran diferenciadas con ácido retinoico durante 9 dias e mantidas 24h con TLQP-62 para comparación de alteracións proteomicas con relación as mesmas celulas na ausencia deste péptido. No estudo proteómico, demostrouse que TLQP-62 induce a expresión de varias proteinas relacionadas con procesos de neuro-desenvolvemento e plasticidade sináptica, como crecemento e comunicación celular (CEP135, CHD5, EIF4H, HNRNPD, PPI1, TUBB), organización do citoesqueleto e crecemento do axón (CALD1, CNN3, DPYSL2, DPYSL3, DYST), metabolismo e biosíntese (COPB2, IDH3A, NAA20, PAFH1B3, PNPO), función mitocondrial (MRPS22), resposta oxidativa (GSTP1, PARK7, SOD1)

e resposta inmunolóxica (NIT2, PCBP1, PGAM2, PSMB2, PSMB3). Estes procesos son fundamentais para a neuroxénese e están disregulados en varias enfermidades mentais e neurolóxicas. Estas alteracións proteomicas explican as alteracións morfolóxicas e a capacidade de TLQP-62 para promover e soportar o crecemento de neuritas. Varias das proteínas identificadas foran asociadas con enfermidades neuropsiquiátricas, como ALB, DPYSL2, DPYSL3, ERP29, GSTP1, IDH3A, PAFAH1B3, PARK7, PGAM2, SOD1, TPI1 ou TUBB. Asín, TLQP-62 regula varias proteinas envolvidas no ciclo celular, inducindo paraxe do ciclo celular e inhibindo a proliferación celular. Outras proteinas están envolvidas no estrés oxidativo e resposta inmune, permitindo a protección celular e sobrevivencia. E aínda, regula proteinas envolvidas no metabolismo e organización do citoesqueleto permitindo o crecemento de dendritas e a neuro-diferenciación celular, contribuíndo para o neurodesenvolvemento e sinaptoxénese.

Para comprender os mecanismos moleculares de TLQP-62 na neuroxénese e neurodiferenciación é necesario determinar o receptor de TLQP-62 que permite a transdución de sinal. Para tal, utilizouse cromatografía de afinidade pola avidina para atrapar o receptor a partir do lisado membranar de celulas SH-SY5Y con biotina-TLQP-62, identificándose o receptor acoplado á proteina G, OR5P3. O mesmo receptor foi tamén identificado en hipocampo humano. Os efectos observados na morfoloxía e proteosoma das celulas SH-SY5Y causados pelo TLQP-62 poderán ocorrer a través da activación deste receptor. Comprobouse a capacidade de TLQP-62 para aumentar os niveis de cAMP num modelo celular que sobreexpresa OR5P3. O cAMP é un mensaxeiro secundario moito importante envolvido en varias vías moleculares de sinalización, activando proteinas quinasa, como PKA, que fosforilan CREB orixinando a transcrición de xenes como BDNF, TrkB e VGF. Estas alteracións terán que ser confirmadas por estudos de inmunodetección en SH-SY5Y tratadas con TLQP-62.

A chaperona HSPA8 foi tamén identificada e a sua capacidade para interactuar con TLQP-62 foi avaliada por análise de redistribución dinámica de masa. Esta chaperona interactúa tamén con TLQP-21. HSPA8 faz parte da familia de HSP70 que interactúa con varias proteinas, sen especificidade, tendo asín diferentes funcións intra e extracelulares. Especificamente, HSPA8 é responsable por conferir a

correcta estrutura a varias proteinas, pero tamén polo seu transporte e distribución, por modular vías de sinalización e de transdución, na inmunidade, autofagia e no estrés celular. HSPA8 é inducida por estrés, hipoxia, exercicio, estróxeno, entre outros. Está presente en toda a celula incluíndo na membrana plasmática, en *lipid rafts*. Transporta proteinas e receptores acabados de sintetizar para a membrana celular, axudando na sua estabilización e na sua activación, como no caso dos receptores de glucocorticoides. HSPA8 pode axudar a estabilizar TLQP-62 e/ou o seu receptor OR5P3. O complexo HSPA8-TLQP-62-OR5P3 pode ser necesario para dar inicio á transdución de sinal, iniciada pelo aumento de cAMP, que activa a vía de sinalización de PKA, inducindo a expresión de CREB, levando á expresión de varias proteinas como BDNF, TrkB ou VGF, e outras proteinas envolvidas na regulación do ciclo celular, na organización do citoesqueleto, no estrés oxidativo e resposta inmunitária.

A estrutura de TLQP-62 foi avaliada utilizándose dicroísmo circular e resonancia magnética nuclear. Determinouse que este neuropéptido ten una estrutura secundaria maioritariamente desorganizada en solución, en transición con unha alfa-hélice, que pode ser estabilizada a diferentes pHs e probabelmente será tamén estabilizada en complexo con HSPA8 ou OR5P3. Futuros estudos estruturais e moleculares son necesarios para mellor comprender os mecanismos de acción de TLQP-62 e de que forma é que un potencial agonista pode ser producido e usado como tratamento para algunhas enfermidades neuropsiquiátricas e neurolóxicas.

O presente estudo contribuín con novos dados sobre os efectos moleculares do TLQP-62 na neurodiferenciacion, con uma avaliación morfolóxica e proteomica. Este péptido é capaz de inducir o crecemento de neuritas, ao inhibir o ciclo celular e a proliferación celular, inducindo a organización do citoesqueleto, o metabolismo enerxético e a biosíntese de neurotransmisores, e aínda promovendo a supervivencia celular a travéz da regulación de xenes mitocondrias e do estrés oxidativo.

Comprender como é que este péptido participa nos procesos sinápticos e de neurodesenvolvemento contribuín para un maior coñecemento do seu envolvemento na fisiopatoloxía das enfermidades neuropsiquiátricas e nun posible mecanismo de tratamento. A identificación de OR5P3 como receptor humano deste neuropéptido

TLQP-62, axudará a desvendar os mecanismos moleculares por detrás destas enfermidades e da neuroxénese. Novos dados sobre a estrutura deste neuropéptido poderán axudar a entender mellor a sua función e como ocorre a sua interacción con o seu receptor e outros ligandos, e proporcionar o desenvolvemento de un posible agonista que poderá ser usado como tratamento.

Como perspectivas futuras para mellor caracterizar o impacto de TLQP-62 na neuroxénese e nas enfermidades neuropsiquiátricas, mais estudos deberán ser realizados. Estudos tendo en conta a via de sinalización activada por OR5P3-TLQP-62 para desvendar cales os intermediarios directos que inducen a expresión de BDNF e outros xenes. Estudos estruturais dos complexos OR5P3-TLQP-62 e HSPA8-TLQP-62 poderán ser realizados de forma a investigar con maior pormenor as súas características estruturais que poderán ter importancia farmacéutica. Asín, poderá ser desenvolvido un agonista para OR5P3 de forma a mimetizar os efectos de TLQP-62 e a poder ser usado como tratamento destas enfermidades neuronais.

Deste estudo concluíse que: o neuropéptido humano TLQP-62 promove a neurodiferenciación de células SH-SY5Y con crecemento de neuritas; TLQP-62 humano é capaz de soportar a neuro-diferenciación de SH-SY5Y inducida por ácido retinoico permitindo a supervivencia das celulas dunha forma semellante á observada con BDNF; TLQP-62 non é capaz de inducir de unha forma eficaz a neurodiferenciación de células silenciadas para VGF; Con o estudo proteomico demostrouse que o TLQP-62 induce a expresión de varias proteinas envolvidas no subdesenvolvemento e sinaptoxénese; Varias desas proteinas están envolvidas na formación do citoesqueleto e no crecemento do axón, no crecemento e comunicación celular, no metabolismo enerxético e na biosíntese de metabolítos, e no estrés oxidativo e resposta inmunitária; TLQP-62 interactúa con OR5P3 na superficie de celulas SH-SY5Y e no hipocampo humano; O TLQP-62 induce un aumento de niveis de cAMP a travéz do OR5P3, indicando que este é o seu receptor; TLQP-62 interactúa tamén con a chaperona HSPA8 na superficie de celulas SH-SY5Y; Pela sua secuencia primaria, prevese que o TLQP-62 forme unha alfa-hélice C-terminal; Estudos de dicroísmo circulan demostrarán que TLQP-62 ten unha estrutura desordenada en solución, que pode ser transitoria con unha alfa-hélice C-terminal.

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