Universitat de Girona

DOCTORAL THESIS

STUDY OF TWO ANALOGUES OF PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE AS NOVEL THERAPEUTIC COMPOUNDS FOR HUNTINGTON'S DISEASE

Irene Solés Tarrés

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Irene Solés Tarrés

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Doctoral Program in Molecular Biology, Biomedicine and Health

Directed and tutorized by Dr. Xavier Xifró Collsamata

Thesis submitted in fulfillment of the requirement to obtain the doctoral degree from the University of Girona

Universitat de Girona

Dr. Xavier Xifró Collsamata, Professor at the Department of Medical Sciences of the University of Girona,

CERTIFY:

That the thesis entitled *Study of two analogues of pituitary adenylate cyclase-activating polypeptide as novel therapeutic compounds for Huntington's disease*, presented by Irene Solés Tarrés to obtain a doctoral degree has been completed under my supervision.

For all intents and purposes, I hereby sign this document.

Dr. Xavier Xifró Collsamata

A l'Aleix,

Per omplir els dies d'humor, alegria i tendresa

Remember that things are not always as they appear to be.

Curiosity creates possibilities and opportunities.

Roy T. Bennett

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Abbreviations

A

A1	Acetyl- [Ala15, Ala20] PACAP38 propylamide
A2	Ac-[Phe(pI)6,Nle17] PACAP38
AC	Adenylate cyclase
ADNF	Activity-dependent neurotrophic factor
ADNP	Activity-dependent neuroprotective protein
ALS	Amyotrophic lateral sclerosis
APP	Amyloid precursor protein
Aβ	Amyloid beta

B

BAC	Bacterial artificial chromosome
BBB	Blood-brain barrier
Bcl-2	B-cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
BNST	Bed nucleus of the stria terminalis

C

CA	*Cornu ammonis*
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
cAMP	3',5'-cyclic adenosine monophosphate
CBP	CREB-binding protein
CI	Confidence interval
CNS	Central nervous system
CREB	cAMP-response element- binding protein
CRTC1	CREB regulator transcription co-activator 1

D

D1R	Dopamine D1 receptor
D2R	Dopamine D2 receptor
DAG	Diacylglycerol
DARPP-32	Dopamine- and cAMP-regulated phosphoprotein 32 kDa
DG	Dentate gyrus
DPP IV	Dipeptidyl peptidase IV

E

EC	Entorhinal cortex
Egr1	Early growth response 1
ERK1/2	Extracellular signal-regulated kinases 1/2

G

GPCR	G protein-coupled receptors
GPe	Globus pallidus internal
GPi	Globus pallidus external

H

HAP1	Huntingtin-associated protein 1
HATs	Histone acetyltransferases
HD	Huntington's disease
HDAC	Histone deacetyltransferases
HTT	Huntingtin

I

iEGs	Immediate early genes
IL-1β	Interleukin 1β
IL-6	Interleukin 6
IP3	1,4,5-trisphpshpate
ITI	Inter-trial interval

K

KA	Kainic acid

L

LTD	Long-term depression
LTP	Long-term potentiation

M

MAGUK	Membrane-associated guanylate kinase
MAPK	Mitogen-activated protein kinase
mHTT	Mutant huntingtin
MMP9	Matrix metallopeptidase 9
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSNs	Medium-sized spiny neurons
MWM	Morris water maze

N

NES	Nuclear export signal
NF-κB	Nuclear factor-kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NLS	Nuclear localization signal
NMDAR	N-methyl-D-aspartic acid receptors

NO	Nitric oxide
NOLT	Novel object localization test
NORT	Novel object recognition test
NRE	Neuron-restrictive silencer element
NRSF	Neuron restrictive silencer factor
NT3	Neurotrophin-3
NT4	Neurotrophin-4

P

p75NTR	p75 neurotrophin receptor
PACAP	Pituitary adenylate-cyclase activating polypeptide
PBS	Phosphate-buffered saline
PD	Parkinson's disease
PI3K	Phosphatidylinositol 3'-OH kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PolyQ	Polyglutamine
PRD	Proline rich domain
PSD-95	postsynaptic density protein 95

Q

QA	Quinolinic acid

R

REST	Repressor element 1-silencing transcription factor

S

SKF	Src family kinases
SNc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
Sp1	Specificity protein 1
STN	Subthalamic nucleus

T

TNF- α	Tumor necrosis factor α
TrkB	Tropomyosin receptor kinase B
T-SAT	T-maze spontaneous alterantion task

V

VIP	Vasoactive intestinal polypeptide

W

WT	Wild-type

Y

YAC	Yeast artificial chromosome

#

3-NP	3-nitropropionic acid
6-OHDA	6-hydroxydopamine

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Summary

Resum

La malaltia de Huntington (MH) és un trastorn genètic neurodegeneratiu sense cura que es caracteritza per provocar símptomes motors, cognitius i psiquiàtrics. La causa genètica de la MH és una expansió anòmala del codó CAG en el gen de la huntingtina (HTT). L'expressió de la HTT mutada (mHTT) provoca l'activació de diferents mecanismes moleculars tòxics que acaben portant a disfunció i neurodegeneració de diferents regions cerebrals. En la MH, la pèrdua de neurones del nucli estriat és una característica clau en el desenvolupament i la progressió de la descoordinació motora, mentre que la disfunció de l'hipocamp s'associa amb els dèficits cognitius. El polipèptid activador de l'adenilat ciclasa (PACAP) és un neuropèptid molt present en el sistema nerviós central i considerat un potencial agent terapèutic perquè s'ha vist que és capaç de protegir les neurones a través de l'activació de tres receptors acoblats a proteïnes G: el PAC1R, el VPAC1R i el VPAC2R. Recentment, el nostre grup ha demostrat que el tractament amb PACAP millora les funcions cognitives i motores d'un model de ratolí transgènic de la MH millorant la funció sinàptica. Tot i així, la capacitat de PACAP per protegir de la toxicitat induïda per la mHTT i la implicació dels diferents receptors en aquesta neuro-protecció encara no ha estat explorada. A més, l'ús farmacològic del PACAP es veu limitat per la seva baixa biodisponibilitat i pel fet que pot causar efectes secundaris, com la taquicàrdia i la hipotensió, quan activa el VPAC1R i VPAC2R a nivell perifèric. Com que diferents estudis suggereixen que el PAC1R és el principal iniciador dels efectes neuro-protectors de PACAP, s'ha proposat que anàlegs de PACAP modificats per presentar més estabilitat i més afinitat pel PAC1R, podrien ser eines terapèutiques prometedores. Per això, el primer objectiu d'aquesta tesi va ser estudiar la capacitat de PACAP per protegir les cèl·lules estriatals de la toxicitat induïda per la mHTT i desxifrar quin paper hi té el PAC1R. I el segon objectiu va ser explorar el potencial terapèutic de dos anàlegs de PACAP per aturar les alteracions cognitives i motores associades amb la MH.

Primer vàrem estudiar l'efecte neuroprotector de PACAP utilitzant el model cel·lular STHdh de la MH. Vàrem trobar que el tractament amb PACAP (10^{-7} M) durant 24 hores protegia les cèl·lules STHdhQ111/Q111 que expressen la mHTT, reduint els nivells de caspasa escindida 3. Comparant l'efecte neuroprotector de PACAP amb el del pèptid intestinal vasoactiu (VIP, 10^{-7} M), un altre neuropèptid amb alta afinitat només pel VPAC1R i el VPAC2R, vàrem observar que l'activació del PAC1R era necessària per activar proteïnes de supervivència com la Akt i les cinases reguladores de senyals extracel·lulars 1/2 (ERK 1/2), així com per induir l'expressió de proteïnes associades amb l'activitat neurotròfica com la proteïna de resposta de creixement (egr1), el c-fos, la proteïna d'unió a CREB (CBP) i el factor neurotròfic derivat del cervell (BDNF). Per altra banda, l'activació dels VPACR a través del tractament amb VIP podia protegir les cèl·lules de l'apoptosi però no era suficient per induir l'expressió i activació de proteïnes neurotròfiques. Així doncs, vàrem concloure que tots els receptors de PACAP participen en la prevenció de la mort cel·lular, però el PAC1R juga un paper clau promovent les proteïnes neurotròfiques, de manera que vam considerar el PAC1R una potencial diana terapèutica en la patologia de la MH.

En la segona part d'aquesta tesi, vam estudiar el potencial terapèutic de dos anàlegs de PACAP: Acetyl- [Ala15, Ala20] PACAP38 propylamide i Ac-[Phe(pI)6,Nle17] PACAP38, als quals ens hem referit com a A1 i A2, respectivament. Les diferents modificacions que presenten aquests anàlegs en la molècula de PACAP, els fa més estables i amb una afinitat augmentada pel PAC1R.

Per tastar les capacitats dels anàlegs en la funció cognitiva, vàrem realitzar una administració intranasal de l'A1 i l'A2 (30 µg/kg/dia) durant dotze dies en el model de ratolí R6/1 de la MH a partir de les 12 setmanes d'edat, quan els dèficits de memòria ja són presents. Els nostres resultats varen demostrar que ambdós anàlegs milloraven la memòria espacial que depèn de l'hipocamp millorant la plasticitat en aquesta àrea. Vàrem demostrar que l'A1 restaurava els dèficits de memòria espacial en la prova del laberint en T (T-MAZE) incrementant la densitat d'espines sinàptiques i de sinapsis

excitatòries a la regió *Cornu Ammonis 1* (CA1) dels animals R6/1. A més, els nostres resultats indiquen que probablement l'A1 millora la plasticitat sinàptica promovent la localització a la membrana postsinàptica dels receptors de N-metil-D-aspartat (NMDAR) i potenciant la seva funció. De manera important, l'administració d'A1 també va reduir el nombre de inclusions de mHTT intranuclears, que és la marca histopatològica de la MH, en totes les regions hipocampals estudiades. Pel que fa a l'A2, vam trobar que millorava la memòria espacial tant en la prova del T-MAZE com en la prova de nova ubicació d'objectes (NOLT) també incrementant el nombre d'espines dendrítiques a la CA1. De manera interessant, vàrem observar que la majoria dels efectes de l'A2 ocorrien al gir dentat (DG), on va incrementar el nombre de sinapsis excitatòries, va induir l'expressió de CREB i va reduir el nombre d'inclusions de mHTT intranuclears.

Finalment, vàrem estudiar la capacitat dels anàlegs de PACAP per millorar la funció motora seguint el mateix patró d'administració explicat anteriorment en els animals R6/1 però iniciant el tractament intranasal a les 18 setmanes d'edat, quan els dèficits motors ja són presents. Els nostres resultats varen demostrar que els anàlegs de PACAP milloren la coordinació motora i l'equilibri dels animals R6/1 avaluades a través de la prova de la barra d'equilibri i la prova del "rotarod". De manera important l'A1 va protegir les neurones espinoses mitjanes, va induir l'activació de la proteïna Akt, relacionada amb la supervivència, i va reduir el nombre d'inclusions intranuclears de mHTT a l'estriat dels animals R6/1. Tanmateix, cap d'aquests efectes va ser detectat en els animals tractats amb l'A2. De fet, no vam poder descriure els mecanismes moleculars iniciats per l'A2 i que promouen la millora del fenotip motor dels R6/1.

Amb tot, els resultats presentats amb aquesta tesi demostren que el PACAP protegeix les cèl·lules estriatals de la toxicitat induïda per la mHTT i que el PACA1R té un paper clau en aquesta acció. A més a més, demostren que l'administració intranasal d'anàlegs de PACAP, com l'A1 i l'A2, és una bona estratègia per combatre els

símptomes cognitius i motors associats a la MH, suggerint que aquests anàlegs podrien ser compostos terapèutics pel tractament de la MH.

Resumen

La enfermedad de Huntington (EH) es un trastorno genético neurodegenerativo sin cura que se caracteriza por provocar síntomas motores, cognitivos y psiquiátricos. La causa genética de la EH es una expansión anómala del codón CAG en el gen de la huntingtina (HTT). La expresión de la HTT mutada (mHTT) provoca la activación de diferentes mecanismos moleculares tóxicos que terminan llevando a la disfunción y neurodegeneración de diferentes áreas cerebrales. En la EH, la perdida de neuronas del núcleo estriado es una característica clave en el desarrollo y la progresión de la descoordinación motora, mientras que la disfunción del hipocampo se asocia con los déficits cognitivos. El polipéptido activador del adenilato ciclasa (PACAP) es un neuropéptido muy presente en el sistema nervioso central y considerado un potencial agente terapéutico por su capacidad de proteger las neuronas a través de la activación de tres receptores acoplados a proteínas G: el PAC1R, el VPAC1R y el VPAC2R. Recientemente, nuestro grupo ha demostrado que el tratamiento con PACAP mejora las funciones cognitivas y motoras de un modelo de ratón transgénico de la EH mejorando la función sináptica. Sin embargo, la capacidad de PACAP para proteger de la toxicidad inducida por la mHTT y la implicación de los diferentes receptores en esa neuroprotección aún no ha sido explorada. Además, el uso farmacológico del PACAP se encuentra limitado por su baja biodisponibilidad y por el hecho que PACAP puede causar efectos secundarios como la taquicardia y la hipotensión cuando activa el VPAC1R y el VPAC2R a nivel periférico. Debido a que diferentes estudios apuntan al PAC1R como principal iniciador de los efectos neuroprotectores de PACAP, se ha propuesto que los análogos de PACAP modificados para tener una mayor estabilidad y afinidad por el PAC1R, podrían ser herramientas terapéuticas prometedoras. Por ello, el primer objetivo de esa tesis fue estudiar la capacidad de PACAP para proteger las células estriatales de la toxicidad inducida por la mHTT y descifrar qué papel tiene el

PAC1R. Y el segundo objetivo fue explorar el potencial terapéutico de dos análogos de PACAP para frenar las alteraciones cognitivas y motoras asociadas a la EH.

En primer lugar, estudiamos el efecto neuroprotector de PACAP usando el modelo celular STHdh de la EH. Encontramos que el tratamiento con PACAP durante 24 horas protegía las células STHdhQ111/Q111 que expresan la mHTT reduciendo los niveles de caspasa escindida 3. Comparando el efecto neuroprotector de PACAP con el del péptido intestinal vasoactivo (VIP, 10^{-7} M), otro neuropéptido con alta afinidad sólo para el VPAC1R y VPAC2R, observamos que la activación del PAC1R era necesaria para activar proteínas de supervivencia como la Akt y las cinasas reguladoras de señales extracelulares 1/2 (ERK 1/2), así como para inducir la expresión de proteínas asociadas con la actividad neurotrófica como la proteína de respuesta de crecimiento (egr1), el c-fos, la proteína de unión a CREB (CBP) y el factor neurotrófico derivado del cerebro (BDNF). Por otro lado, la activación de los VPACR a través del tratamiento con VIP podía proteger las células de la apoptosis, pero no era suficiente para inducir la expresión y actividad de proteínas neurotróficas. Así pues, concluimos que todos los receptores de PACAP participan en la prevención de la muerte celular, pero el PAC1R juega un papel clave en la activación de proteínas neurotróficas. Por eso, consideramos el PAC1R una potencial diana terapéutica en la patología de la EH.

En la segunda parte de esa tesis, estudiamos el potencial terapéutico de dos análogos de PACAP: Acetyl- [Ala15, Ala20] PACAP38 propylamide y Ac-[Phe(pI)6,Nle17] PACAP38, a los que nos hemos referido como A1 y A2, respectivamente. Las diferentes modificaciones que presentan esos análogos en la molécula hacen que sean más estables y con una afinidad aumentada hacia el PAC1R.

Para probar las capacidades de los análogos en la función cognitiva, realizamos una administración intranasal del A1 y el A2 (30 µg/kg/día) durante doce días en el modelo de ratón R6/1 de la EH a partir de las 12 semanas de edad, cuando los déficits de memoria ya son presentes en esos animales. Nuestros resultados demostraron que

ambos análogos mejoraban la memoria espacial dependiente de hipocampo mejorando la plasticidad en esa área. Demostramos que el A1 restauraba los déficits de memoria espacial en la prueba del laberinto en T (T-MAZE) incrementando la densidad de espinas sinápticas y de sinapsis excitatorias en la región *Cornu ammonis 1* (CA1) de los animales R6/1. Además, nuestros resultados indican que probablemente el A1 mejora la plasticidad sináptica promoviendo la localización de los receptores de N-metil-D-aspartato (NMDAR) en la membrana postsináptica y potenciando su función. De forma importante, la administración de A1 también redujo el nombre de inclusiones de mHTT intranucleares, que es la huella histopatológica de la EH, en todas las regiones hipocampales estudiadas. Por otro lado, el análogo A2, mejoró la memoria espacial tanto en la prueba del T-MAZE como en la de nueva ubicación de objetos (NOLT) incrementando el número de espinas dendríticas en la CA1. De forma interesante, observamos que los efectos de A2 sucedían principalmente al giro dentado (DG), dónde incrementó el número de sinapsis excitatorias, indujo la expresión de CREB y redujo el número de inclusiones de mHTT intranucleares.

Finalmente estudiamos la capacidad de los análogos para mejorar la función motora siguiendo el mismo patrón de administración explicado anteriormente en los animales R6/1 pero iniciando el tratamiento intranasal a las 18 semanas de edad, cuando los déficits motores ya son presentes. Nuestros resultados demostraron que los análogos de PACAP mejoran la coordinación motora y el equilibrio de los animales R6/1 evaluadas con la prueba de barra de equilibrio y la prueba del "rotarod". De forma importante, el A1 protegió a las neuronas espinosas medianas, indujo la activación de la proteína Akt, relacionada con la supervivencia, y redujo el nombre de inclusiones de mHTT intranucleares en el núcleo estriado de los animales R6/1. Sin embargo, no encontramos esos efectos en los animales tratados con el A2. De hecho, no pudimos describir los mecanismos moleculares iniciados por el A2 que promueven la mejora del fenotipo motor de los R6/1.

Con todo, los resultados presentados en esta tesis demuestran que el PACAP protege las células estriatales de la toxicidad inducida por la mHTT y que el PAC1R tiene un papel clave en esta acción. Además, demuestran que la administración intranasal de análogos de PACAP, como el A1 y el A2, es una buena estrategia para combatir los síntomas cognitivos y motores asociados a la EH, sugiriendo que esos análogos podrían ser compuestos terapéuticos para el tratamiento de la EH.

Summary

Huntington's disease (HD) is a neurodegenerative genetic disorder with no effective treatment characterized by motor, cognitive, and psychiatric alterations. The genetic cause of HD is an abnormal expansion of the CAG codon repeat in the huntingtin (HTT) gene. The expression of the resulting mutant HTT (mHTT) activates different toxic molecular mechanisms leading to the dysfunction and degeneration of different brain regions. The loss of striatal neurons is a key feature in the development and progression of motor discoordination, whereas hippocampal dysfunction has been associated with cognitive deficits in HD. Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide widely distributed throughout the central nervous system considered a potential therapeutic agent for protecting neurons through the activation of three G protein-coupled receptors: PAC1R, VPAC1R, and VPAC2R. Recently, our laboratory demonstrated that PACAP improves cognitive and motor functions in a transgenic mouse model of HD by enhancing synaptic function. However, the effects of PACAP on mHTT-induced cell toxicity, and the involvement of PACAP receptors in neuroprotection have not yet been explored. In addition, the pharmacological use of PACAP is limited by its low bioavailability and secondary effects, such as tachycardia and hypotension, owing to the activation of peripheral VPACRs. As PAC1R is suggested to be the main effector of the neuroprotective effect of PACAP, more stable PACAP analogues with higher PAC1R affinity have been proposed as promising therapeutic tools. Therefore, the first aim of this thesis was to study the capacity of PACAP to protect striatal cells from mHTT-mediated toxicity and

to decipher the involvement of PAC1R. The second aim was to explore the therapeutic potential of two synthetic PACAP analogues against the cognitive and motor alterations that occur in HD.

First, we studied the neuroprotective effects of PACAP using the STHdh cellular model of HD. We found that PACAP treatment (10^{-7} M) for 24 hours protected STHdhQ111/Q111 cells expressing mHTT from apoptosis by reducing cleaved caspase-3 levels. By comparing the protective effect of PACAP and vasoactive intestinal peptide (VIP, 10^{-7} M), another neuropeptide with high affinity only for VPAC1R and VPAC2R, we observed that PAC1R activation was necessary for the activation of the pro-survival proteins Akt and extracellular signal-regulated kinases (ERK 1/2), as well as for the expression of neurotrophic proteins, such as early growth response (egr1), c-fos, cAMP-response element-binding protein (CREB)-binding protein (CBP), and brain-derived neurotrophic factor (BDNF). In contrast, although specific VPACR activation by VIP could protect cells from apoptosis, its stimulation was not sufficient to enhance the expression and activation of neurotrophic proteins. Overall, we established that all PACAP receptors may participate in preventing cellular death, but PAC1R plays a key role in promoting neurotrophic effects. Thus, we considered PAC1R a promising therapeutic target in HD pathology.

In the second part of this thesis, we studied the therapeutic potential of two analogues of PACAP: Acetyl- [Ala15, Ala20] PACAP38 propylamide and Ac-[Phe(pI)6,Nle17] PACAP38, referred to as A1 and A2, respectively. The different modifications in the PACAP molecule make these analogues more stable than PACAP while displaying greater selectivity for PAC1R.

To test the capacity of analogues on cognitive function, we performed an intranasal administration of A1 or A2 (30 µg/kg/day) for twelve days in the R6/1 mouse model of HD from 12 weeks of age, when memory deficits were present. Our results demonstrate that both analogues improved hippocampal-dependent spatial memory

deficits in R6/1 mice by enhancing hippocampal plasticity. We showed that A1 restored spatial memory deficits evaluated by the T-MAZE test by increasing dendritic spine density and the number of excitatory synapses in the R6/1 *Cornu ammonis 1* (CA1) region. In addition, our results indicated that A1 may improve synaptic plasticity by promoting postsynaptic density localization of N-methyl-D-aspartic acid receptors (NMDAR) and potentiating their function. Remarkably, A1 administration also reduced the number of intranuclear inclusions of mHTT, the histopathological hallmark of HD, in all hippocampal regions studied in the R6/1 mice. In contrast, A2 improved spatial memory deficits evaluated by the T-MAZE test and novel object location test (NOLT) by increasing the density of dendritic spines in the CA1. Interestingly, we observed the main effects of A2 in the dentate gyrus (DG), where it increased the number of excitatory synapses, induced CREB activation, and reduced intranuclear inclusions of mHTT.

Finally, we studied the therapeutic ability of PACAP analogues to improve motor function following the same administration design explained previously, but starting intranasal treatment at 18 weeks of age, when motor deficits were already present. Our results demonstrate that PACAP analogues improve coordination and balance in R6/1 mice evaluated by the balance beam and the rotarod tests. Importantly, we found that A1 protected medium spiny neurons, induced the activation of the pro-survival pathway Akt, and reduced the number of intranuclear mHTT aggregates in the striatum of R6/1 mice. These effects were not shared with those of A2. In fact, the molecular mechanisms initiated by A2 underlying the recovery of the motor phenotype remain unclear.

Altogether, the results presented in this thesis show that PACAP protects against mHTT-induced toxicity and that PAC1R have a key role in this action. In addition, our outcomes indicate that intranasal administration of PACAP analogues, such as A1 and A2, fights motor and cognitive deficits associated with HD, suggesting that PACAP analogues could be therapeutic compounds for the treatment of HD.

CHAPTER I: INTRODUCTION

1. Huntington's disease

Huntington's disease (HD) is a hereditary neurodegenerative disorder characterized by progressive degeneration of specific regions of the central nervous system (CNS) that leads to a combination of motor, cognitive, and behavioral symptoms. HD was first described in 1872 by George Huntington, who named it Chorea (derived from the ancient Greek word Choreia, meaning dance) on account of the discoordinated movements of those who are affected by it (Huntington, 1872). George Huntington recognized three marked peculiarities accompanying this type of chorea: its hereditary nature, the tendency for insanity and suicide of the patients, and its manifestation as a grave disease in adult life (Huntington, 1872). His original description remains valid. However, Huntington's chorea was renamed Huntington's disease because of the increasing description of non-motor alterations in people suffering from HD (Wexler, 2013). Currently, there is no cure for this disorder, and existing treatments only lessen some motor and psychiatric symptoms, with no capacity to stop the course of neuropathology. This condition is devastating not only to patients, but also to their families.

1.1. Epidemiology

HD is endemic to all populations, but reviews of epidemiology indicate that its worldwide prevalence varies widely. A systematic review published by Rawlins and collaborators in 2016 reviewed all studies on the prevalence of HD between January 1930 and June 2015. This study showed the lowest prevalence rates among blacks in South Africa (0.02, 95% confidence interval [CI] 0–0.5 per 100,000) and Zimbabwe (1.00, 95% CI 0.48–1.84 per 100,000). In Asian populations, the prevalence rate was also low (0.40, 95% CI 0.36–0.44 per 100,000). In contrast, the highest prevalence was found among Caucasians (Australia, Western Europe, and North America), which was 9.71, 95%, CI 9.32–10.12 per 100,000. Importantly, the authors suggested that this

variation can in part be attributed to differences in case-ascertainment of diagnostic criteria, but they affirmed that there is consistent evidence of lower incidence in Asian populations (Rawlins et al., 2016). These findings are consistent with those of previous systematic meta-analyses (Pringsheim et al., 2012).

1.2. Natural history and disease progression

Neurodegeneration in HD is progressive and selective, which characterizes its clinical course. HD progression can be divided into three clinical phases with variable durations: pre-manifest, prodromal, and manifest (Ross et al., 2014). During the pre-manifest stage, no signs or symptoms related to HD are observed. Next, patients can experiment a prodromal phase for many years, during which they suffer from subtle motor and cognitive alterations and behavioral changes (Kirkwood et al., 1999). The manifest period is characterized by definitive motor signs suggestive of HD, accompanied by cognitive difficulties and emotional alterations (Ghosh and Tabrizi, 2018). These symptoms progress slowly and are clinically divided into five stages: early, mild, moderate, late, and severe (Ross et al., 2014). The average age of HD manifestation is 45 years, and patients usually die after 10-20 years (Ross et al., 2014) (Figure 1). In approximately 4–10% of all HD cases, the onset of disease occurs before the age of 21 years (Quarrell et al., 2012). This is referred to as juvenile HD (Quarrell et al., 2012). The progression of juvenile HD is faster, and survival is shorter than that of adult-onset HD (Bakels et al., 2021).

Generally, HD is diagnosed based on a confirmed family history or a positive genetic test result and the onset of motor disturbances. Although the course of disease is variable, in adult-onset HD, motor symptoms are characterized by involuntary motor abnormalities known as early chorea (Rothlind et al., 1993; Smith et al., 2000). Progression to mild chorea is characterized by oculomotor disturbances, brisk muscle stretch reflexes, and diminished rapid alternating movement (Kirkwood et al., 2001). Finally, functionally limiting chorea appears as voluntary movement abnormalities and

motor deficits such as dystonia and athetosis (Ghosh and Tabrizi, 2018). Cognitive deficits appearing early in the disease are mainly related to executive functioning, progressive deficits in hippocampal-dependent spatial memory, and impaired visuospatial abilities (Lawrence et al., 1996; Harris et al., 2019). Later, cognitive decline leads to dementia, which has been suggested to be consequence of cortical damage identified in HD patients (Martinez-Horta et al., 2020). Behavioral abnormalities are heterogeneous and show no clear progression during HD. Depression and apathy are very common and may occur several years before motor abnormalities begin, suggesting that they may be part of the disease process (Julien et al., 2007). Moreover, people with HD tend to have a lower body mass index due to altered metabolism (Robbins et al., 2006; Duan et al., 2014) and may present a disruption in sleep and circadian rhythms, possibly due to hypothalamic dysfunction (Petersén and Björkqvist, 2006; Morton, 2013). Although the symptomatology in the early and middle stages is variable, HD in the late stages is well-defined. In the late stage of HD, patients are functionally incapacitated, with global dementia and severe limitations in voluntary movements, characterized by bradykinesia, spasticity, dysarthria, dysphagia, incontinence, and full dependence on daily activities (Folstein, 1989; Kirkwood et al., 2001; Ghosh and Tabrizi, 2018).

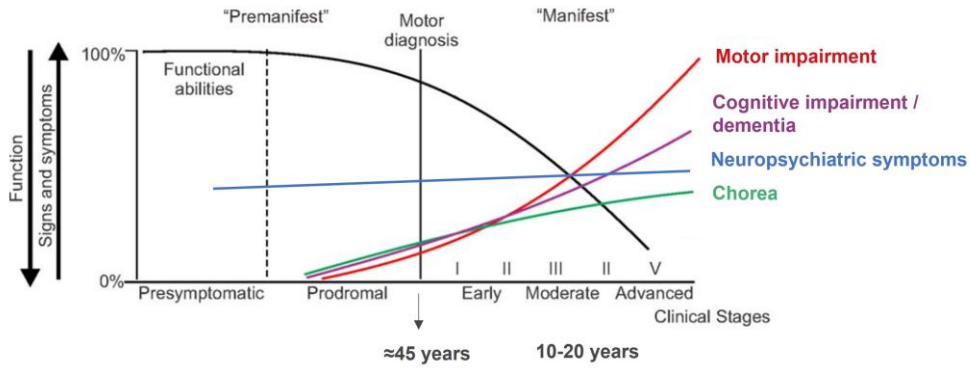


Figure 1. Schematic timeline of motor, cognitive and neuropsychiatric symptomatology of HD. HD development is progressive and symptomatology that appears early continues and become worsen as the pathology proceed, until the loss of all functional abilities. The average of HD manifest is 45 years, and the progression can be divided in 5 stages named early, mild, moderate, late, and advanced. The patients usually die within 10 to 20 years after the manifest.

1.3. Etiology

1.3.1. Genetic mutation

The causative gene of Huntington's disease is known as the huntingtin (HTT) gene because of its name assigned to the protein. This gene was mapped in 1983 to the short arm of human chromosome 4 in the 4p16.3 region (Gusella et al., 1983). However, it was not until 1993 that an aberrant mutation was described. This mutation consists in an increase in the number of repetitions of three nucleic acids (C, A, and G) in the coding region of the first exon of the HTT gene, resulting in an expansion of the polyglutamine (PolyQ) domain (The Huntington's Disease Collaborative Research Group., 1993). The normal length of the CAG triplet typically ranges from 17 to 19 repeats (Kremer et al., 1994), but when the number of repeats increases to 40 or more, it results in the expression of the disease. Although HD is a genetic disorder with autosomal dominant inheritance, the number of CAG repeats is variable and fundamental in HD clinical expression because CAG repeats determine the penetrance of the disease (Langbehn et al., 2004).

In non-HD individuals, the CAG triplet in the HTT gene is normally repeated ≤ 26 times (Semaka et al., 2006). However, part of them can present an allele called "intermediate allele" with a range between 27 and 35 CAG repetitions. These individuals do not develop HD symptoms (Semaka et al., 2006). However, they may be at risk of passing an allele in the HD-causing range to their offspring because of CAG tract instability (Semaka et al., 2006; Semaka and Hayden, 2014). When the CAG repeat size is 36 or more, we are talking about pathogenic HD-causing alleles. These alleles are called

reduced-penetrance HD-causing alleles when their range or repetitions range from 36 CAG to 39 CAG. In these cases, individuals are at risk for HD, but may not develop symptoms (Kay et al., 2016). In contrast, when an individual present full-penetrance HD-causing alleles with 40 or more CAG repeats, the manifestation of the disease is assumed (Caron et al., 2020) (Figure 2).

The nature of the genetic defect in the HTT gene explains not only the penetrance of the disease, but also the variability in age of onset (Duyao et al., 1993). A significant inverse correlation between the number of CAG repeats and HD age of onset has been described. Thus, individuals with longer CAG repeats typically present an earlier age of onset (Langbehn et al., 2004, 2010). In line with this statement, individuals who suffer from juvenile HD usually have more than 60 CAG repeats in the HTT gene, and some patients with pediatric onset can have more than 80 repeats (Telenius et al., 1993; Fusilli et al., 2018) (Figure 2). Additionally, CAG repeat length has also been shown to predict the age of death and the rate of deterioration of motor and cognitive functions increases with larger CAG repeats (Aziz et al., 2009; Keum et al., 2016; Chao et al., 2017).

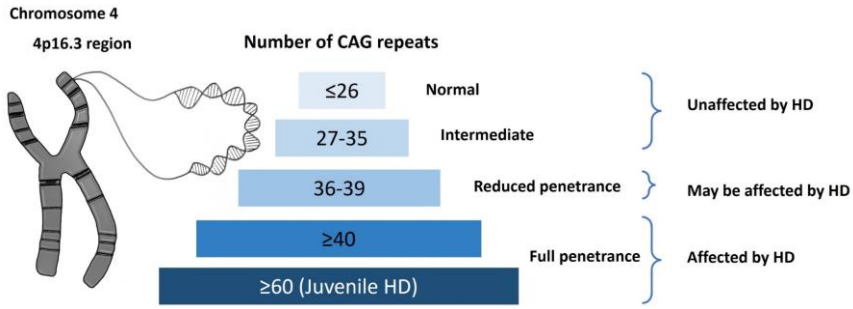


Figure 2. Localization of HTT gene and penetrance of the disease. The HTT gene is located in the short arm of human chromosome 4 at the 4p16.3 region. The causative mutation of HD is an increase in the number of CAG repetitions in the coding region of the first exon of the HTT gene. Clinical expression of HD depends on the number of CAG repeats. The length of the tract can be classified as normal, intermediate, reduced, or full penetrance. Normal and intermediate CAG lengths result in individuals unaffected by HD. Individuals may express HD when the number of repeats is between 36 and 39. However, when the number of repetitions is ≥ 40, the individuals become completely affected by HD.

1.3.2. The huntingtin protein

Huntingtin (HTT) is the protein product of HTT gene. This protein has a large size (>350 kDa MW) and its primary amino acid sequence, shown schematically in Figure 3, provides information about its normal function and the possible molecular mechanisms altered by the mutation. The N-terminal region has been extensively studied because it contains an expandable PolyQ domain. The polyQ domain is preceded by 17 amino acids conforming the N17 region. This domain forms an amphipathic α-helix structure that plays an important role in the degradation and subcellular localization of HTT (Atwal et al., 2007; Maiuri et al., 2013; Rockabrand et al., 2007). PolyQ domains of normal length are suggested to mediate the binding of factors bearing polar residues, such as transcription factors (Perutz et al., 1994). Importantly, polyQ can reduce protein solubility (Fiumara et al. 2010). However, HTT is a soluble protein. In mammals, the polyQ tract is followed by a proline rich domain (PRD) (Tartari et al., 2008a). Interestingly, PRD is thought to confer solubility to the HTT protein (Ignatova and Gierasch, 2006). Additionally, PRD may be important for stabilizing the structure of polyQ stretch and can also mediate protein-protein interactions (Dehay and Bertolotti, 2006). Following this N-terminal fragment, HTT contains several tandem clusters of HEAT, a protein tandem repeat of ~40 amino acids named after the four proteins in which it was first detected: HTT, elongation factor 3, protein phosphatase 2A, and TOR1. HEAT repeat domains may function as scaffolds for numerous protein complexes and mediate important intermolecular and intramolecular interactions (Takano and Gusella, 2002; Tartari et al., 2008; Palidwor et al., 2009). This suggests that HTT can adopt various three-dimensional conformations depending on the intramolecular interactions. HTT also has motifs related to its localization, such as the nuclear export signal (NES) and nuclear localization signal (NLS), indicating that HTT function can be associated with both the nucleus and cytoplasm (Xia et al., 2003; Saudou and Humbert, 2016). Additionally, HTT has several post-translational sides by which its activity, localization and interaction with other

proteins can be modified (Saudou and Humbert, 2016). These post-translational modifications include phosphorylation, acetylation, ubiquitination, sumoylation, and palmitoylation (Steffan et al., 2004; Atwal et al., 2007; Thompson et al., 2009; Maiuri et al., 2013). Importantly, HTT can be also subjected to proteolysis at different sites by a wide variety of proteases (Gafni and Ellerby, 2002; Lunkes et al., 2002; Hermel et al., 2004; Kim et al., 2006; Ratovitski et al., 2009). Thus, a lot of proteolytic sites are distributed thorough the HTT. These proteolytic sites are PEST domains, regions rich in proline (P), glutamic acid (E), serine (S) and threonine (T) (Warby et al., 2008).

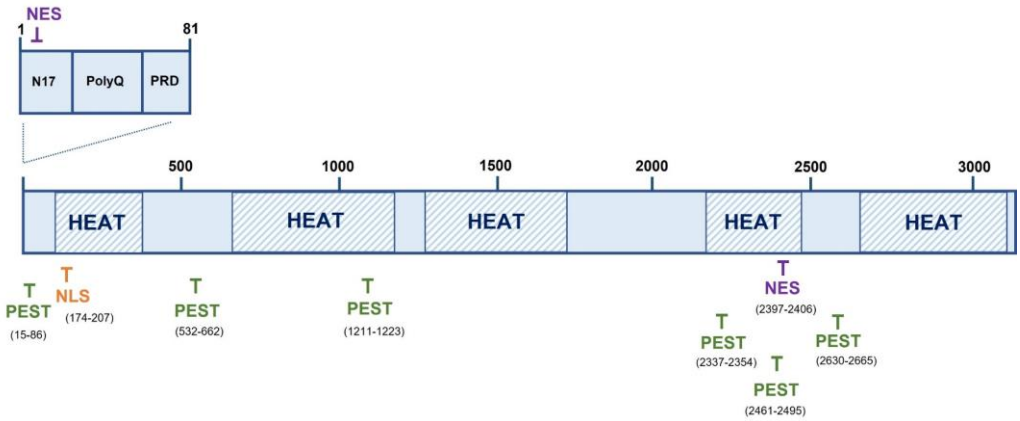


Figure 3: Schematic representation of the primary amino acid sequence of human HTT. The diagram shows the amino acid positions for the N17 domain, polyglutamine tract (PolyQ), proline rich domain (PRD), HEAT domains, nuclear export signals (NES), nuclear localization signals (NLS), and proteolytic sites that are PEST, rich in proline (P), glutamic acid (E), serine (S), and threonine (T). Adapted from Saudou and Humbert (2016).

Functions of huntingtin

HTT protein is found at different levels in most human and murine tissues, and its expression begins early during development and persists in adulthood (Marques Sousa and Humbert, 2013). It is well known that HTT is important for the formation of the nervous system, as it has an important role in several processes such as differentiation and neuronal survival (Saudou & Humbert, 2016). In fact, HTT knockout embryos display defects in gastrulation resulting in embryonic lethality (Duyao et al., 1995; Nasir

et al., 1995; Zeitlin et al., 1995). In parallel, some studies indicate that HTT is also important for neuronal homeostasis and survival in adult CNS (Liu and Zeitlin, 2017). Some of the molecular processes in which HTT participates in the CNS are summarized below.

- **Transport of organelles in axons and dendrites within neurons:** HTT interacts with molecular motor machinery directly with dynein or through Huntingtin-associated protein 1 (HAP1) (Gunawardena et al., 2003; Gauthier et al., 2004; McGuire et al., 2006; Caviston et al., 2007; Colin et al., 2008; Twelvetrees et al., 2010). By these interactions HTT facilitates the transport increasing the velocity and coordinating the direction. Some organelles transported by HTT are synaptic precursor vesicles (Zala et al., 2013), autophagosomes (Wong and Holzbaur, 2014), endosomes, and lysosomes (Caviston et al., 2011; Liot et al., 2013). Importantly, HTT also controls the transport of brain-derived neurotrophic factor (BDNF)-containing vesicles (Gauthier et al., 2004)
- **Cell division**: HTT is abundant in dividing cells, in which it is localized at the spindle poles, mitotic spindles, and astral microtubules. During mitosis in neuronal and non-neuronal cells, HTT is important for the assembly and orientation of proteins at the spindle point (Gutekunst et al., 1995; Godin et al., 2010; Elias et al., 2014).
- **Endocytosis, vesicle recycling, and endosomal trafficking** HTT interacts with several proteins to form larger complexes involved in endocytosis and vesicle recycling (Sittler et al., 1998; Modregger et al., 2003). It has been suggested to act as a support protein at the beginning of endocytosis, helping in membrane coating and invagination (Engqvist-Goldstein et al., 2001; Waelter et al., 2001; Legendre-Guillemin et al., 2002).
- **Autophagy:** HTT regulates the transport of autophagosomes along axons (Wong and Holzbaur, 2014). Moreover, mutated HTT (mHTT) has been proposed to abnormally activate autophagy pathways (Martin et al., 2014).

- **Transcriptional regulation.** HTT is largely cytoplasmic but is also present in the nucleus. In the polyQ tract, there are motifs that can act as transcriptional regulatory domains, allowing binding between transcription factors and transcriptional regulators. The cAMP-response element- binding protein (CREB) -binding protein (CBP)(Steffan et al., 2000), specificity protein 1 (Sp1) (Dunah et al., 2002), and nuclear factor-kappa-light-chain-enhancer of activated B cells (NF-κB) (Takano and Gusella, 2002) are transcription factors that bind to HTT. Through these interactions, HTT can potentiate or inhibit the transcription factors and repressors that modulate gene transcription.
- **Synaptic communication**: HTT has also been shown to interact with proteins in presynaptic and postsynaptic terminals, playing a role in the transport and release of synaptic vesicles as well as synaptic function (Metzler et al., 2003; Twelvetrees et al., 2010). For example, HTT interaction with postsynaptic density protein 95 (PSD-95), an essential protein for post-synaptic density organization and dendrite morphology, directly influences its localization and activity (Sun et al., 2001; Parsons et al., 2014).
- **Cell survival:** Different *in vitro* and *in vivo* studies have shown that HTT expression protects cells against apoptosis and excitotoxicity (Leavitt et al., 2000, 2001a; Leavitt et al., 2001, 2006). One of the molecular mechanisms associated with this neuroprotection is HTT-mediated inhibition of pro-apoptotic proteins, such as caspase-3 and -9 (Rigamonti et al., 2000, 2001; Zhang et al., 2006).

1.3.3. Mutated huntingtin and its contribution to HD pathology

Expanded CAG repeats in exon 1 of HTT gene are translated into an expanded polyQ stretch in mutant HTT (mHTT) (The Huntington's Disease Collaborative Research Group., 1993). The presence of this abnormally expanded polyQ stretch has been speculated to promote mHTT loss-of-function, leading to the neuropathology of HD.

Accordingly, the different processes in which HTT takes part have been found to be affected in the context of HD. For instance, deficits in BDNF are suggested to be due to the inability of mHTT to promote its transport in vesicles (Gauthier et al., 2004). The normal function of HTT in autophagy is also lost in the presence of this mutation (Martinez-Vicente et al., 2010). Additionally, it was demonstrated that with only knockout of HTT in the adult mouse brain, progressive neurodegeneration reminiscent of HD occurs. This evidence supports the idea that part of HD neuropathology may be due to the inability of mHTT to properly perform its normal functions. However, the abnormal polyQ stretch may also induce a gain of toxicity, independent of loss of function. One of the most obvious characteristics of mHTT gained by the mutation is the tendency of the protein to form aggregates, both in the nucleus and cytoplasm of neurons (DiFiglia et al., 1997; Scherzinger et al., 1997), which correlates with the length of polyQ expansion (Scherzinger et al., 1999). Longer polyQ tracts promote increased proteolytic cleavage. Thus, mHTT is cleaved by proteases, generating N-terminal fragments containing an abnormal polyQ stretch (DiFiglia et al., 1997; Sieradzan et al., 1999; Hoffner et al., 2007). The process of aggregation starts when mHTT monomers stick together to form oligomeric species, which gradually increase in size while losing their solubility (McGowan et al., 2000; Poirier et al., 2002; Legleiter et al., 2010). Many essential proteins are susceptible to be sequestered within mHTT inclusions leading to loss of their normal function. CBP (Cong et al., 2005; Jiang et al., 2006) and wild-type (WT) HTT (Busch et al., 2003) are examples of proteins reported to be repressed and consequently inhibited by mHTT. Importantly, some authors hold that mHTT aggregates are neutral bioproducts of the pathogenic process, while others suggest that they are neuroprotective. This assumption is based on the hypothesis that inclusions of mHTT may reduce the level of more toxic soluble forms of the protein (Saudou et al., 1998; Miller et al., 2010). Currently, there is still debate regarding neurotoxicity versus neuroprotection offered by aggregates. However, the inhibition of mHTT aggregates formation has been shown to have beneficial effects in a

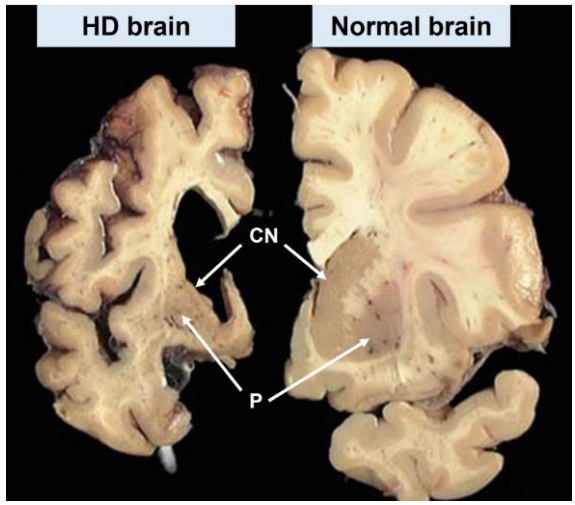
Drosophila model of polyQ disease (Kazantsev et al., 2002) and in a transgenic mouse model of HD (Sánchez et al., 2003).

1.4. Neuropathology

Neuropathological studies have shown that brain abnormalities in HD appear before evident symptoms, are progressive, and result in approximately 25% of brain loss in advanced HD (Figure 4A) (Sharp and Ross, 1996; Halliday et al., 1998). Importantly, in this neurodegenerative process, some brain areas become affected earlier and more severely than others. The most prominent neurodegeneration in HD occurs in the striatal part (caudate nucleus and putamen in humans) of the basal ganglia, leading to a large enlargement of the lateral ventricles (Figure 4A) (Vonsattel et al., 1985). A system for grading the severity of HD based on macroscopic and microscopic criteria related to striatal morphology was proposed in 1995 by neuropathologist Jean-Paul Vonsattel. This system establishes five grades of ascending severity from 0 to 4, with grades correlating closely with the degree of clinical disability (Vonsattel et al., 1985). The figure 4B schematically shows the neuropathological grading of HD with summarized macroscopic and microscopic particularities for each grade (Reiner et al., 2011).

Apart from striatal neuropathology, marked neuronal loss has also been observed in the deep layers of the cerebral cortex. Additionally, variable degrees of atrophy and/or neuronal loss have been detected in the globus pallidus, hippocampus, amygdala, thalamus, subthalamic nucleus, substantia nigra, and cerebellum (Rosas et al. 2003). Here, we focus on the HD-associated neuropathology occurring in the striatum and hippocampus, the two brain regions in which dysfunction mainly contributes to the HD-related behavioral impairments studied in this thesis.

(A)



(B)

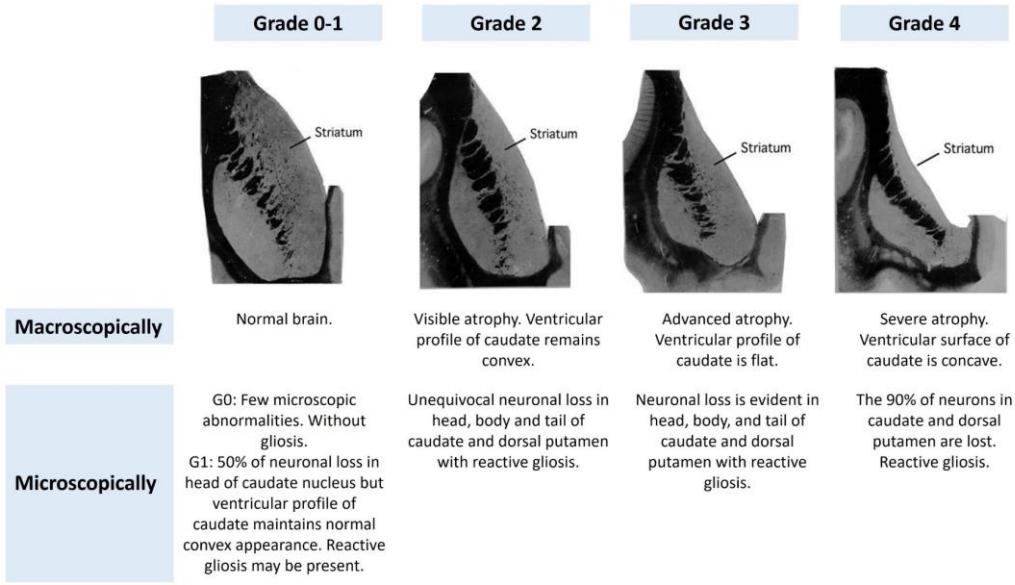


Figure 4. Neuropathology in HD. (A) Postmortem human coronal brain sections showing a normal brain and an advanced HD brain. In the HD brain it can be observed the degeneration occurring in caudate nucleus (CN) and putamen (P) as well as the cortical atrophy and the enlargement of the lateral ventricle. Modified from Reiner et al., 2011. **(B)** Schematic illustrations showing the Vonsattel grading system of striatal degeneration in HD. Macroscopic and microscopic particularities of each grade are summarized. Modified from Reiner et al., 2011.

1.4.1. Basal ganglia pathology and associated motor disturbances

As mentioned above, the striatum is the most affected structure in HD patients. Importantly, the striatum is part of the basal ganglia, a group of subcortical nuclei responsible for motor control as well as other roles such as motor learning, executive functions and behaviors, and emotions (Lanciego et al., 2012). The term basal ganglia in the strictest sense refers to nuclei embedded deep in the brain hemispheres: the striatum, which includes the caudate and putamen, and the globus pallidus, which is divided into two parts: the internal (GPi) and external (GPe). However, the function of the basal ganglia depends on its strong association with its named related nuclei: the subthalamic nucleus (STN), the substantia nigra, which is divided into the reticular part (SNr) and the compact part (SNc), and the pedunculopontine nucleus (Figure 5A) (Lanciego et al., 2012).

The basal ganglia and related nuclei are heavily interconnected to facilitate and modulate the movement. The main input of the basal ganglia is the striatum, which receives information mainly from cortical (glutamatergic), thalamic (glutamatergic), and nigral (dopaminergic) origins. (Lanciego et al., 2012). About 90-95% of neurons in the striatum are projecting neurons, known as medium-sized spiny neurons (MSNs), and the remaining 5-10% are interneurons (Kreitzer, 2009). Although all MSNs are GABAergic inhibitory neurons, distinct populations of MSNs that project to different output structures shape the two principal pathways of the basal ganglia: direct and indirect pathways. These pathways have opposing effects on the activity of the thalamus, which modulates cortical activation. Thus, direct pathway activation facilitates movement, whereas indirect pathway activation is associated with stopping or halting movement (Albin et al., 1989; Kravitz et al., 2010; Freeze et al., 2013). It is thought that their simultaneous but balanced activity allows the execution of controlled movements (Cui et al., 2013).

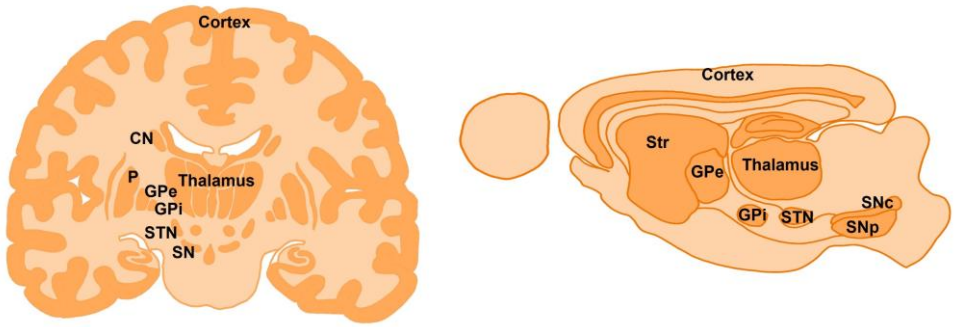
In the direct pathway, when a cortical glutamate excitatory signal activates the striatum, MSNs project to the SNr and the GPi. Because neurons in the SNr and GPi constantly inhibit the thalamus, its inhibition results in disinhibition of the thalamus, which in turn projects its excitatory signals to the motor cortex, facilitating movement. Conversely, in the indirect pathway, when cortical glutamate excitatory signals arrive in the striatum, MSNs project to the GPe. Neurons from the GPe exert an inhibitory action on STN; thus, the inhibition of GPe results in the disinhibition of STN. In turn, the STN sends excitatory projections to the SNr and GPi, which, as mentioned, sends inhibitory signals to the thalamus. Hence, in the indirect pathway, SNr and GPi are activated by STN, which results in strong inhibition of the thalamus. Consequently, fewer excitatory signals from the thalamus arrive at the motor cortex, resulting in finalization of the movement (Figure 5B) (Alexander and Crutcher, 1990; Lanciego et al., 2012; Plotkin and Goldberg, 2019).

Importantly, in addition to their distinct projections, MSNs of direct and indirect pathways are characterized at the molecular level. MSNs from the direct pathway express dopamine D1 receptors (D1R), whereas MSNs from the indirect pathway express dopamine D2 receptors (D2R) (Albin et al. 1989; Valjent et al. 2009; Gerfen and Surmeier 2011). Remarkably, the D1R and D2R receptors are associated with distinct G proteins, leading to opposite molecular responses. Consequently, the nigrostriatal dopaminergic pathway originating from the SNc activates the D1R-expressing direct pathway while inhibiting the D2R-expressing indirect pathway (Gerfen et al., 1990; Gerfen and Surmeier, 2011). Thus, dopamine released from the nigrostriatal pathway modulates the activity of the direct and indirect striatal pathways, facilitating voluntary movements or inhibiting unwanted movements (Figure 5B) (Crossman, 2000).

Multiple studies have indicated that an imbalance in the activity of the direct and indirect pathways of the basal ganglia contributes to the motor symptoms of HD. Excessive glutamate and dopamine release have been observed in the early stages of

HD (André et al., 2011a; Koch and Raymond, 2019; Joshi et al., 2009). This is thought to encourage the activation of the direct pathway, promoting excessive disinhibition of the thalamus (Joshi et al., 2009; André et al., 2010, 2011b). Moreover, some authors hold that MSNs of the indirect pathway are the most vulnerable and the first to degenerate, which also contributes to the disinhibition of the thalamus at the early stages (Reiner et al., 1988; Deng et al., 2004). This results in overactivation of the cortex, which is believed to underlie choreatic symptoms in the first stages of HD (André et al., 2010). In contrast, a lack of glutamate and dopamine signaling has been observed at a later stage, which causes deactivation of the direct pathway (Johnson et al., 2006; Joshi et al., 2009; André et al., 2010, 2011b; Callahan and Abercrombie, 2011; Rothe et al., 2015; Koch and Raymond, 2019). Additionally, MSNs of the direct pathway degenerate at the late stages of HD (Deng et al., 2004; Reiner and Deng, 2018; Plotkin and Goldberg, 2019). Thus, both pathways are affected, leading to overall inhibition of the thalamus and cortex. It is when chorea is replaced by hypoactivity and muscle rigidity (Figure 5B) (André et al., 2010; Reiner A. and Deng Y., 2018).

(A)



(B)

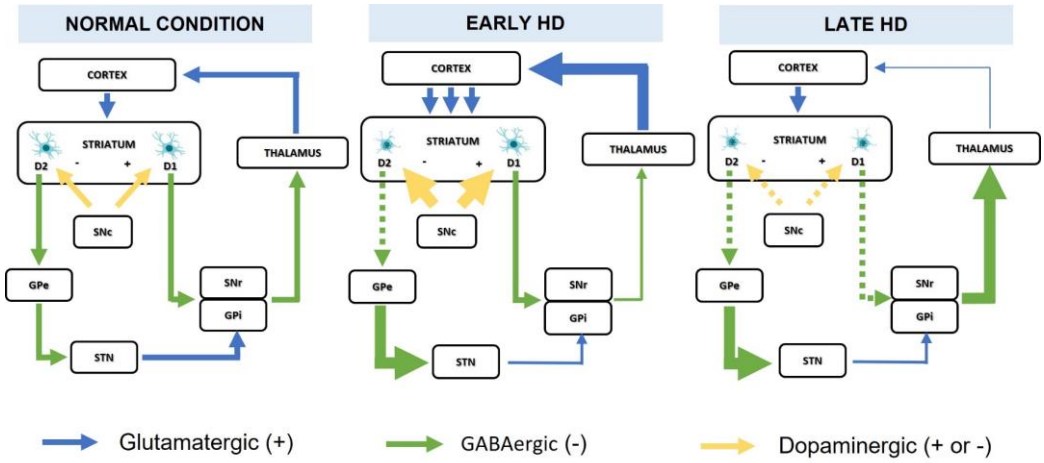


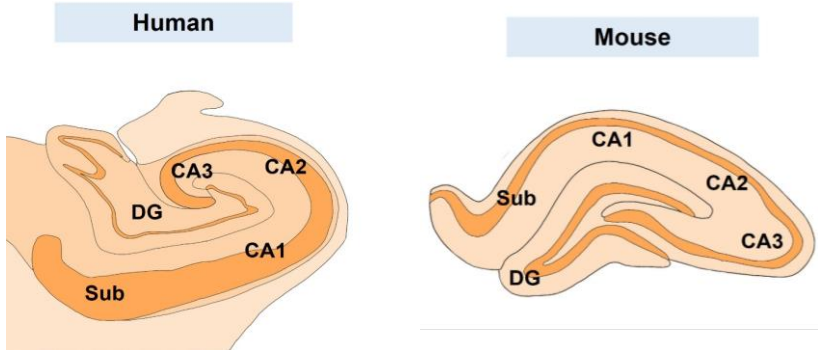
Figure 5. Localization of the basal ganglia in the brain and striatal pathology in HD. (A) Schematic representation of the localization of the basal ganglia and related nuclei in a human coronal brain section and in a mouse sagittal brain section. Basal ganglia are formed by the caudate nucleus (CN), putamen (P), globus pallidus internal (GPi), and globus pallidus external (GPe). The related nuclei of the basal ganglia are the subthalamic nucleus (STN), substantia nigra (SN), which is divided into the reticular part (SNr), compact part (SNc), and pedunculopontine nucleus. Thalamus and cortex are also represented **(B)** Simplified schematic representation showing the basal ganglia circuitry under normal conditions and during HD. Please refer to the text for this information. A larger thickness in arrows indicates larger activity of the pathways, and thinner arrows indicate less activity. Discontinuity in the arrows indicates the main affectation of the pathway. Adapted from André et al. 2010.

1.4.2. Hippocampal pathology and associated cognitive disturbances

The hippocampus is a complex brain structure that is embedded deep into the temporal lobe. It forms the central axis of the limbic system, and is indispensable for memory acquisition, consolidation, and spatial navigation (Squire 2003, 2004; Moser et al. 2008). The hippocampus is part of the allocortex and is separated from the neocortex by the subiculum and entorhinal cortex (EC). Some anatomists divide it into the proper hippocampus (*Cornu ammonis*; CA), dentate gyrus (DG) and subiculum. Entire set is called as hippocampal formation (Anand and Dhikav, 2012). Importantly, CA is divided in CA1, CA2, and CA3, and the principal neurons in these areas are

pyramidal cells. In contrast, the principal neurons in the DG are the granule cells (Figure 6A). These different regions process their inputs during learning to generate an output that contributes to distinct aspects of memory encoding (Anand and Dhikav, 2012; van Strien et al., 2009; Basu and Siegelbaum, 2015). The principal input of the hippocampus is the perforant path, which originates from the superficial layers of the EC and reaches CA1 through the two principal circuit systems in the hippocampus: the monosynaptic and trisynaptic circuits (van Strien et al., 2009). In the monosynaptic circuit, axons from layer III of the EC project directly to pyramidal neurons in CA1 and CA3. In contrast, axons from layer II of the EC initiate a trisynaptic path. In this case, excitatory projections from the EC arrive at the DG through the perforant path. From the DG, mossy fiber projections excite pyramidal neurons from CA3, which in turn excite pyramidal neurons from CA1 through the Schaffer collateral pathway. CA1 projects to the subiculum, which in turn sends the main output of the hippocampus back to the EC (Anand and Dhikav, 2012; Basu and Siegelbaum, 2015). Following this principal loop, hippocampus process and temporarily stores new memories prior to their permanent storage in the cortex (Figure 6B).

(A)



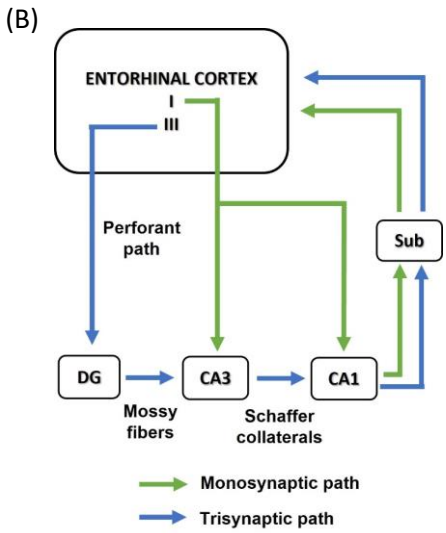


Figure 6. Hippocampal anatomy and hippocampal basic pathways. (A) Schematic representation of the principal elements of hippocampal formation. Hippocampal formation is divided into *Cornu ammonis* (CA1, CA2, CA3), dentate gyrus (DG), and subiculum (Sub). **(B)** Simplified schematic representation showing hippocampal intrinsic circuitry. Monosynaptic and trysinaptic paths allow hippocampal formation to process and temporarily store new memories. Please refer to the text for this information.

Accumulating evidence suggests that hippocampal dysfunction can contribute to cognitive impairment in HD. Hippocampal volume is slightly reduced in the early stages of the disease (Rosas et al., 2003), and a specific neuronal loss has been detected in the CA1 region of HD patients (Spargo et al., 1993). Additionally, several studies have found that HD patients present alterations in learning and memory tasks, which are modulated in part by the hippocampus (Lawrence et al., 2000; Berrios et al., 2002; Lemiere et al., 2004; Montoya et al., 2006; Begeti et al., 2016; Glikmann-Johnston et al., 2019; Harris et al., 2019). Importantly, it has recently been demonstrated that hippocampal volume correlates with spatial memory impairment in HD (Glikmann-Johnston et al., 2021). Evidence supporting hippocampal-mediated cognitive deficits in HD comes from the R6/1, R6/2, YAC128, and HdhQ7/Q111 mouse models of the disease, which display impaired hippocampal-dependent spatial learning and navigation (Lione et al., 1999; Lüesse et al., 2001; Nithianantharajah et al., 2008; Brooks et al., 2012a; Giralt et al., 2012b). Changes in hippocampal connectivity due to HD remain poorly understood. However, several pathophysiological processes described in the hippocampus of HD mouse models have been associated with cognitive deficits. Examples of these alterations are aberrant

synaptic transmission (Murphy et al., 2000; Brito et al., 2014; Giralt et al., 2017; Dargaei et al., 2019), reduced neurogenesis (Gil et al., 2005; Phillips et al., 2005; Lazic et al., 2006), and accumulation of mHTT aggregates (Murphy et al., 2000; Ramaswamy et al., 2007).

1.5. Mouse and cellular models for Huntington's disease research

Generating models capable of recapitulating the pathogenesis and progression of HD is essential for the development and evaluation of new therapeutic strategies. Since the identification of the causative gene of HD, multiple animal models have been generated in a variety of animal species. However, mouse *(mus musculus)* models still dominate the research field, probably because of the close genetic relationship between rodents and humans, in addition to low cost, rapid generation, and reliable methods of genetic manipulation (Sosa et al., 2012).

The first models used to study HD were non-genetic models, typically obtained by inducing striatal cell death either by excitotoxic mechanisms or by disruption of the mitochondrial machinery. Injection of kainic acid (KA) (Coyle and Schwarcz, 1976; McGeer and McGeer, 1976), quinolinic acid (QA) (Beal et al., 1991; Ferrante et al., 1993), and 3-nitropropionic acid (3-NP) (Beal et al., 1993; Brouillet et al., 1993)was used to mimic the pattern of striatal neurodegeneration seen in patients of HD mo(Ramaswamy et al., 2007). However, the study of HD pathogenesis in these models was limited because there was no mHTT expression.

Emerging molecular technologies have enabled the development of genetic murine models that attempt to capture the hereditary nature of HD. To date, more than twenty different HD genetic mouse models have been developed. In general, HD genetic mouse models can be classified into two main categories: transgenic and knock-in. Transgenic mice result from the random insertion of the mHTT gene, or part

of it, into the mouse genome. Alternatively, in knock-in mice, the mutation is inserted specifically into the mouse HTT gene.

As previously mentioned, the transgenic mouse models can be for the 5' region of the human HTT gene, generating an N-terminal fragment of HTT protein. The N-terminal transgenic lines developed include R6/1, R6/2, and N171-82Q. The R6/2 is the most used. The fragment of the human HTT gene inserted into R6/2 mice is only exon 1 and expresses approximately 144 CAG repeats (Mangiarini et al., 1996). The substantial number of repeats in the R6/2 model results in a very aggressive behavioral phenotype with cognitive and motor symptoms appearing very early, at the age of 6-9 weeks (Stack et al., 2005). R61/1 is very similar to R6/2, but the inserted fragment expresses approximately 116 repeats, making the behavioral phenotype relatively mild (Mangiarini et al., 1996). This model has been used in this thesis and is discussed in more detail in 1.4.1 section. N171-82Q expresses an N-terminally truncated human mHTT regulated by a mouse prion promoter, which results in mHTT expression throughout the mouse brain but is restricted to neurons and not glia (Schilling et al., 1999). In this model the fragmented mHTT has 82 polyQ repeats, which results in a late onset of symptomatology (Schilling et al., 1999). On the other hand, transgenic models can express mutant versions of the full-length HTT protein. In this case, models were generated by introducing the human mutant HTT gene in the form of yeast artificial (YAC) or bacterial artificial (BAC) chromosomes. YAC mouse strains contain 72 or 125 CAG repeats (Hodgson et al., 1999; Slow et al., 2003; Pouladi et al., 2012) whereas BAC mice contain 97 CAA and CAG mixed repeats (Gray et al., 2008). YAC and BAC mice display progressive behavioral deficits between 2 and 6 months of age, which become more pronounced at 12 months (Pouladi et al., 2012). In contrast to N-terminal transgenic mice, full-length mouse models gradually develop the disease over many months and show normal survival times. Therefore, they are suitable for long-term therapeutic studies (Ramaswamy et al., 2007).

Transgenic mouse models have certain limitations. First, they contain multiple copies of the HTT gene, two normal murine copies and one mutated human copy. In addition, as HTT expression is regulated by an artificial promoter, the spatial and temporal control of expression is different from that of the endogenous mouse HTT promoter. Finally, random insertion of the human HTT gene in the mouse genome may interfere with the normal function of other genes not related to HD. All these concerns have led to the creation of knock-in mouse models, which result from the replacement of the HTT mouse gene with a mutant human copy that contains an expanded CAG region (Ramaswamy et al., 2007). Thus, knock-in mice have a mutation inserted into the mouse HTT gene and can be homozygous or heterozygous for the mutation. Several Knock-in models with different repeat lengths have been developed using two strategies. The first, consists of the homologous recombination of the endogenous HTT gene with a chimeric human/mouse fragment containing the CAG repeat tract. This approach allowed the obtention of the HdhQ92 (Wheeler et al., 1999), HdhQ111 (Wheeler et al., 1999), CAG140 (Menalled et al., 2003) and zQ175 (Menalled et al., 2012) models, among others. The second and most recently strategy is inserting only an expanded CAG repeat tract in the CAG repeat region of the murine HTT gene, also by homologous recombination. The HdhQ150 (Lin et al., 2001), HdhQ200 (Heng et al., 2010), and HdhQ250 (Jin et al., 2015) models were obtained using this technique. In general, larger CAG repeat stretches in these mice result in earlier, quicker, and more severe symptomatology (Wheeler et al., 2000; Lin et al., 2001; Menalled, 2005; Heng et al., 2010; Brooks et al., 2012b; Menalled et al., 2012; Hölter et al., 2013; Jin et al., 2015; Yhnell et al., 2016). Although knock-in mice represent a more precise genetic model of HD, their behavioral deficits are not as pronounced as those observed in transgenic mice, and it takes much longer to develop them.

It is important to mention that the generation of cell models of HD has also helped to better understand the molecular mechanisms that contribute to HD pathogenesis. These cell lines are typically developed from genetic HD animal models (Lunkes and

Mandel, 1998; Trettel et al., 2000). In this thesis, it has been used the mouse striatal cell line STHdh, which is described in detail in 1.4.2 section.

1.5.1. The transgenic R6/1 mouse model

As mentioned previously, the R6/1 mouse model is a transgenic HD model with exon 1 of the human HTT gene, with approximately 116 CAG repeats randomly inserted in its genome. In R6/1 mice, the expression of mHTT is under the control of the human HTT promoter and is expressed in all cells at 31% of the expression levels seen in the endogenous mouse genome. R6/1 mice have significant body weight loss at 22 weeks and survive for more than 12 months (Ferrante, 2009).

R6/1 mice have a marked behavioral phenotype that includes motor and cognitive deficits. Deficits in both recognition and spatial memory are present at 12 weeks of age, as established using the novel object recognition test (NORT), novel object localization test (NOLT), T-maze spontaneous alternation task (T-SAT) and Morris water maze (MWM) (Giralt et al., 2011b, 2013; Brooks et al., 2012b; Saavedra et al., 2013; García-Forn et al., 2018). Importantly, these cognitive deficits are usually associated with hippocampal alterations. On the other hand, the onset of motor symptoms is reported to occur between 12 and 20 weeks of age (Van Dellen et al., 2008; Brooks et al., 2012c; Cabanas et al., 2020). However, some authors have found deficits in the static rotarod at 10 weeks (García-Lara et al., 2018). Other behavioral disturbances detected in R6/1 mice include stereotypic movements at the ages of 24 and 34 weeks, an increase in resting time (García-Lara et al., 2018), gait abnormalities measured by footprint analysis and hindlimb clasping behavior, and lower levels of anxiety than WT mice at late ages (Naver et al., 2003).

Referring to neuropathology, in R6/1 mice there is a reduction in cerebral volume and neuronal atrophy (Naver et al., 2003). R6/1 mice show decreased dopamine- and cyclic adeonisine monophosphate (cAMP)-regulated phosphoprotein (DARPP-32) expression, indicating cellular dysfunction in the striatum (van Dellen et al., 2000).

Cellular mHTT inclusions appear throughout the R6/1 brain, including the striatum and hippocampus, and their number increases with age (Naver et al., 2003). Interestingly, it has been found correlations between the level of mHTT aggregation and behavioral changes in R6/1 mice (Cabanas et al., 2020).

1.5.2. The STHdh cellular model

STHdh cell lines were originally established in the laboratory of Dr. Marcy MacDonald from the striatal primordia of the HD knock-in mouse model and WT littermates (Trettel et al., 2000). STHdhQ7/Q7 cells stably express full-length WT HTT with 7 glutamines whereas STHdhQ111/Q111 cells stably express full-length mHTT with 111 glutamines at endogenous levels (Trettel et al., 2000).

This cellular model has been used for years in various studies to characterize the molecular disturbances caused by the presence of mHTT. Some alterations found in HD animal models have also been observed in these cells, such as elevated levels of activated Akt (Gines et al., 2003a), decreased protein kinase A (PKA)-CREB signaling (Gines et al., 2003b), reduced levels of the , tropomyosin receptor kinase B (TrkB) (Brito et al., 2013), alterations in the Ras/ mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinases 1/2 (ERK1/2) pathway (Ginés et al., 2010), and increased sensitivity to oxidative damage (Xifró et al., 2008; Ginés et al., 2010), among others.

1.6. Molecular mechanisms involved in Huntington's disease neuropathology

Complex molecular alterations are involved in the neuronal dysfunction and neuronal death that occurs in HD. As no curable treatments are available, there is a need to better understand these molecular alterations underlying HD neuropathology to develop new pharmacological treatments against appropriate targets. In that section,

it has been summarized some of molecular alterations showed in both HD patients and different HD models.

1.6.1. Transcriptional dysregulation

Transcriptional dysregulation has been well established as a pathological process in HD and occurs very early in the pathogenesis. Consistent changes in the gene expression of neurotransmitters, neurotrophins and their receptors, as well as proteins related to stress-response pathways and cell death, have been described in the HD human brain and in experimental disease models, some of them even before the onset of the symptoms (Hodges et al., 2006; Kuhn et al., 2007; Seredenina and Luthi-Carter, 2012; Vashishtha et al., 2013; Hervás-Corpión et al., 2018; Gallardo-Orihuela et al., 2019; Yildirim et al., 2019; Malla et al., 2021). Several mechanisms involving the presence of the mHTT have been proposed to explain the transcriptional dysregulation in HD.

One hypothesis is the aberrant protein-protein interaction between mHTT and transcription-related proteins. The polyQ stretch at the N terminus of mHTT promotes its interaction with glutamine-rich activation domains of different transcription factors and coactivators such as Sp1, its coactivator TAFII130, and CBP (Steffan et al., 2000; Dunah et al., 2002). This interaction can involve the polyQ repeats of mHTT from insoluble aggregates, which cause the sequestration of these transcription factors (Kazantsev et al., 1999; Steffan et al., 2000; Cong et al., 2005), or the polyQ repeats of the soluble form of mHTT, which perturb the interaction of these transcription factors with other transcription-related proteins or their target DNA (Nucifora et al., 2001; Dunah et al., 2002; Jiang et al., 2006). In both cases, the resulting consequence is the inhibition of transcriptional activity. Accordingly, the expression of Sp1 and CREB target genes has been found to be downregulated in both *in vitro* and *in vivo* models of HD (Nucifora et al., 2001; Dunah et al., 2002; Sugars et al., 2004; Chen-Plotkin et al., 2006). Importantly, CREB mediates the transcription of genes critical for neuronal survival and plasticity, such as BDNF (Sakamoto et al., 2011) (Figure 7, 1).

On the other hand, mHTT loses the capacity to interact with negative transcriptional regulators and consequently cause the downregulation of some neuronal genes. This is the case for the transcriptional repressor element 1 -silencing transcription factor/neuron restrictive silencer factor (REST/NRSF). It has been shown that WT HTT interacts with REST/NRSF in the cytoplasm, preventing its nuclear translocation and binding to a DNA sequence element called RE1 or neuron-restrictive silencer element (RE1/NRE) (Zuccato et al., 2003, 2007). In HD, the mHTT interaction with REST/NRSF is weaker, leading to its nuclear entry, which binds to the RE1/NRE sites and suppresses the transcription of BDNF and other neuronal genes (Zuccato et al., 2003, 2007; Bithell et al., 2009) (Figure 7, 2).

Interestingly, mHTT has also been implicated in structural alterations of chromatin. The activities of histone acetyltransferases (HATs) and histone deacetyltransferases (HDACs) play a critical role in the structure of DNA to allowing or suppressing its transcription, respectively (Yang and Seto, 2007). In HD, mHTT disrupts the HAT and HDACs balance, resulting in a general transcriptional repression (Sadri-Vakili and Cha, 2006; Sharma and Taliyan, 2015) (Figure 7, 3). For example, in addition to its coactivator function, CBP can act as a HAT. In HD, mHTT blocks the HAT activity of the CBP, which has been associated with cognitive deficits (Steffan et al., 2001; Korzus et al., 2004; Giralt et al., 2012a).

Finally, mHTT can also dysregulate transcription by directly interacting with the genomic DNA (Benn et al., 2008). It has been proposed that mHTT can interact with specific promoters and dysregulate the transcription of different genes at the early stages of HD (Benn et al., 2008; Hogel et al., 2012; Pearl et al., 2020) (Figure 7, 4).

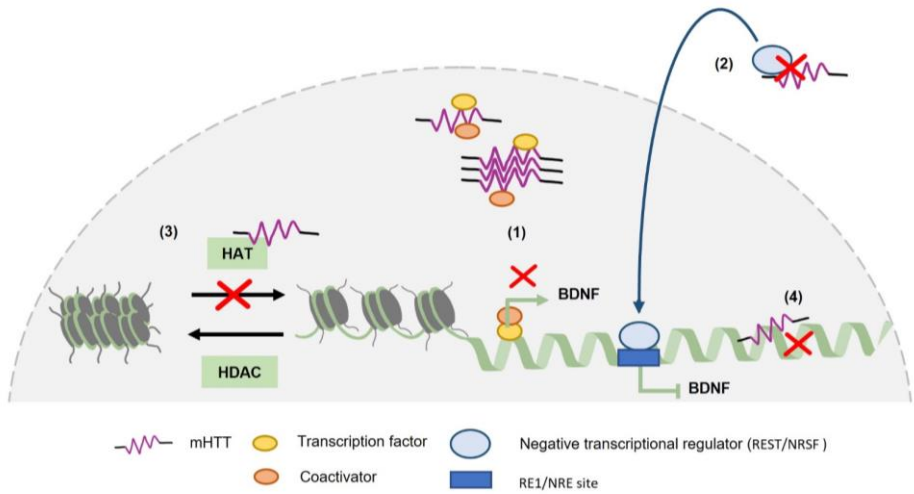


Figure 7: Mechanisms of transcriptional dysregulation in HD. Schematic diagram showing the transcriptional processes disrupted by mutant huntingtin (mHTT). **(1)** Soluble and mHTT aggregates show aberrant interactions with positive transcriptional regulators, causing the downregulation of genes critical for neuronal survival and plasticity, including brain-derived neurotrophic factor (BDNF). **(2)** mHTT fails to interact with negative transcriptional regulators in the cytoplasm, which results in their translocation to the nucleus and the suppression of the transcription of BDNF and other neuronal genes. **(3)** mHTT disrupts histone acetyltransferase (HAT) activity, resulting in transcriptional repression. **(4)** mHTT dysregulates transcription by directly interacting with genomic DNA.

1.6.2. Synaptic dysfunction

Very early in the progression of HD, patients and mouse models display changes in neuronal activity, synaptic transmission, and plasticity in cortico-striatal connections (Cepeda et al., 2003; Starling et al., 2005; Joshi et al., 2009; Raymond et al., 2011; Bunner and Rebec, 2016; Smith-Dijak et al., 2019; Fernández-García et al., 2020) as well as in the different areas of the hippocampus (Bliss and Collingridge, 1993; Murphy et al., 2000; Giralt et al., 2011b, 2012b, 2012a; Miguez et al., 2015; Anglada-Huguet et al., 2016a; Wilkie et al., 2020). Owing to the high abundance of excitatory synapses in

these two regions, molecular elements involved in excitatory synaptic transmission have become of great interest in the study of HD.

N-methyl-D-aspartic acid receptors (NMDARs) are glutamate-gated ion channels that are widely expressed in the CNS playing key roles in excitatory synaptic transmission (Traynelis et al., 2010). NMDARs are tetrameric complexes formed by different subunits that can be divided into three subtypes: GluN1, GluN2A-D, and GluN3A-B (Cull-Candy et al., 2001; Chatterton et al., 2002; Mayer, 2004). The combination of these subunits in NMDARs determines their functional properties such as the permeability of Ca^{2+}, its localization in the synaptic membrane, and their interaction with intracellular proteins (Kornau et al., 1995; Cull-Candy et al., 2001; Pérez-Otaño et al., 2001, 2006; Waxman and Lynch, 2005; Paoletti and Neyton, 2007; Gambrill and Barria, 2011; Shi et al., 2011; Shipton and Paulsen, 2014). In most cases, NMDARs are composed of two GluN1 and two GluN2 subtypes (Lee et al., 2014). When glutamate binds to NMDARs on the postsynaptic membrane, it promotes the flow of Ca^{2+} into the cell and increases excitability (Shi et al., 2011). Importantly, NMDARs promote the induction and preservation of synaptic activity, regulation of gene expression, and participate in plasticity processes, such as long-term potentiation (LTP) and long-term depression (LTD) (Daw et al., 1993; Lau and Zukin, 2007; Rebola et al., 2010; Hunt and Castillo, 2012).

In early stages of HD, it has been described an increase of responsiveness of NMDARs in striatal projection neurons (Cepeda et al., 2001; Laforet et al., 2001; Zeron et al., 2002; Starling et al., 2005). This augmented activity of NMDARs, added to abnormal high concentrations of glutamate observed in HD (Behrens et al., 2002; Estrada-Sánchez and Rebec, 2012; Rebec, 2013), is thought to underlie the excitotoxicity process that causes MSNs neurodegeneration (Zeron et al., 2002, 2004; Shehadeh et al., 2006). Interestingly, as the disease progresses, NMDAR function is reduced, resulting in resistance to excitotoxicity in later stages of the disease (Hansson et al.,

1999, 2001; Graham et al., 2009; Joshi et al., 2009). Consistent with this, studies in transgenic mouse models of HD have demonstrated abnormal changes in NMDAR subunit expression at different life stages in both striatum and hippocampus, leading to aberrant NMDAR activity (Cha et al., 1999; Cepeda et al., 2001; Giralt et al., 2017; Fão et al., 2022b). Additionally, some authors have pointed out an increase of extrasynaptic NMDAR in HD, promoting neuronal dysfunction and death (Milnerwood et al., 2010; Zhou et al., 2013) (Figure 8).

Interestingly, other postsynaptic proteins have been found to be affected in HD and suggested to be involved in synaptic dysfunction. This is the case for PSD-95, the most studied scaffolding protein in the membrane-associated guanylate kinase protein (MAGUK) family. PSD-95 has been shown to bind to the C-terminal tail of GluN2 subunits, stabilizing and modulating NMDAR function (Kornau et al., 1995; Funke et al., 2005; Xu, 2011; Won et al., 2016; Compans et al., 2021). Protein levels of PSD-95 are strongly downregulated in HD patients and in the striatum and hippocampus of HD mouse models (Torres-Peraza et al., 2008; Anglada-Huguet et al., 2014; Fourie et al., 2014; Murmu et al., 2015; Giralt et al., 2017), which has been suggested to participate in the mislocalization and aberrant functionality of NMDARs (Torres-Peraza et al., 2008) (Figure 8).

The NMDAR function and localization are also modulated by post-translational modifications, such as phosphorylation (Chen and Roche, 2007). Specifically, the activity of Src and Fyn, two proteins from the Src family kinases (SFKs), has been linked to the increase in synaptic activity as well as NMDAR current and surface expression (Salter and Kalia, 2004; Rajani et al., 2021). Importantly, Fyn-mediated phosphorylation of tyrosine 1472 stabilize the GluN2B-composed NMDARs in the post-synaptic membrane, controlling synaptic plasticity (Goebel-Goody et al., 2009). In HD, abnormally decreased levels of GluN2B phosphorylation at tyrosine 1472 have been found to facilitates their movement to extra synaptic sites (Gladding et al., 2012; Fão et al., 2022b) (Figure 8). Additionally, SKFs activation and protein levels are decreased

in several human and mouse models of HD, and it has recently been found that its restoration restabilizes NMDARs localization, phosphorylation, and function in striatal neurons from YAC128 mice (Fão et al., 2022a, 2022b).

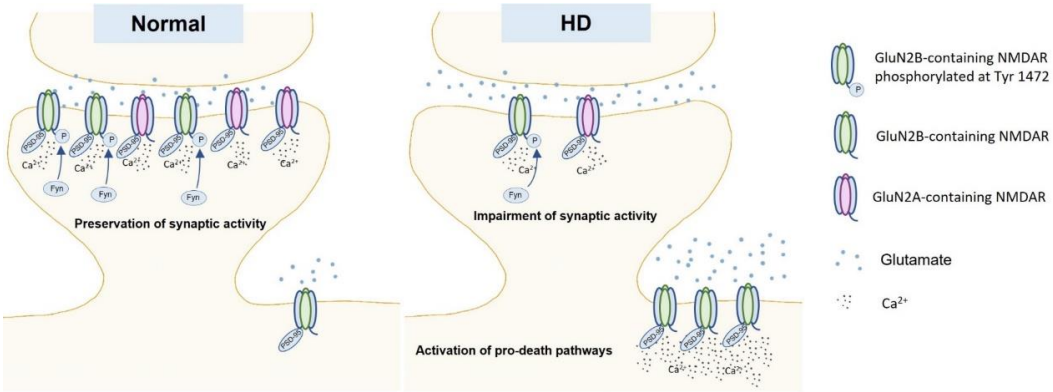


Figure 8: Mechanisms underlying synaptic dysfunction in HD. Schematic diagram showing the synaptic impairment described in HD. Under normal conditions, N-methyl-D-aspartic acid receptors (NMDAR) are situated in the synaptic membrane, stabilized by postsynaptic density 95 (PSD-95) protein and the phosphorylation of the GluN2B subunit at tyrosine 1472 mediated by Fyn protein. In the presence of glutamate, NMDARs are activated, allowing the preservation of synaptic activity. In the context of HD, decreased protein levels of PSD-95 and Fyn are associated with a reduction in NMDAR at the synaptic membrane leading to the impairment of synaptic activity. In addition, GluN2B-containing NMDAR receptors are concentrated in the extra synaptic membrane, where the increase in glutamate levels and responsiveness of NMDARs leads to the activation of pro-death pathways, causing excitotoxicity in neuronal cells.

1.6.3. Neurotrophic dysfunction

Neurotrophic factors enhance neuronal differentiation, survival, and function (Huang and Reichardt, 2003). Among neurotrophic factors, BDNF is considered the major regulator of synapsis and neuronal survival (Arancio and Chao, 2007; Liu et al., 2008). BDNF is expressed and synthesized by both neurons and glial cells (Pöyhönen et al., 2019), and exerts its actions through interaction with its specific cellular receptor, TrkB (Chao, 2003; Sasi et al., 2017). Once BDNF binds to TrkB, ligand-mediated dimerization

of the complex occurs at the cell surface. This is followed by autophosphorylation of specific tyrosine residues in the cytoplasmic domain, which promotes the activation of three interconnected intracellular cascades: phosphatidylinositol 3'-OH kinase (PI3K)/Akt, MAPK/ERK, and phospholipase C (PLC) /phosphokinase C (PKC) (Chao, 2003; Huang and Reichardt, 2003; Sasi et al., 2017). Specifically, the activation of the tyrosine 515 residue triggers the PI3K/Akt and MAPK/ERK pathways, exerting neurotrophic functions such as survival, growth, and differentiation (Brunet et al., 2001; Patapoutian and Reichardt, 2001) whereas the phosphorylation of the tyrosine 816 residue leads to the activation of the PLC/PKC pathway, allowing the enhancement of synaptic plasticity (Minichiello et al., 2002; Chao, 2003) (Figure 9). Importantly, BDNF, and particularly its immature form, pro-BDNF, can interact with the p75 neurotrophin receptor (p75NTR). The activation of p75NTR enhances the Jun cascade signalling, which is related to apoptosis (Reichardt, 2006), and the activation of nuclear factor-kappa-light-chain-enhancer of activated B cells (NF-κB), which contribute to neuronal inflammation (Hamanoue et al., 1999) (Figure 9).

The presence of mHTT disrupts BDNF neurotrophic signalling in several brain regions involved in HD neuropathology. The molecular mechanisms underlying this impairment are deficits in BDNF and alterations in the expression or activity of its receptors. Decreased BDNF mRNA and protein levels have been observed in the brains of HD patients and HD models (Figure 9). In patients undergoing HD, reduced levels of BDNF are found in the caudate nucleus, putamen, and cortex (Ferrer et al., 2000; Zuccato et al., 2001, 2008). In HD models, a decrease in BDNF levels has been found in the cortex, striatum, hippocampus, and cerebellum (Duan et al., 2001; Zuccato et al., 2001, 2005; Luthi-Carter et al., 2002; Hermel et al., 2004; Spires et al., 2004a; Ginés et al., 2006; Lynch et al., 2007; Gharami et al., 2008; Diekmann et al., 2009; Giralt et al., 2009; Simmons et al., 2011; Cabezas-Llobet et al., 2018). It has been suggested that these reductions in BDNF protein levels are a consequence of both mHTT-mediated transcriptional dysregulation and vesicular transport impairment. (Steffan et al., 2001;

Gauthier et al., 2004; Zuccato et al., 2007; Yu et al., 2018). The biological effects of BDNF depend not only on its availability but also on the expression and activity of its transmembrane receptors, TrkB and p75NTR. Importantly, different studies have shown that TrkB levels are reduced in striatal and hippocampal neurons expressing mHTT (Ginés et al., 2006; Zuccato et al., 2008; Brito et al., 2013; Simmons et al., 2013; Miguez et al., 2015; Pérez-Sisqués et al., 2022). In addition, other studies have uncovered important impairments in TrkB signalling (Ginés et al., 2010; Plotkin et al., 2014; Anglada-Huguet et al., 2016b; Barriga et al., 2017). On the other hand, p75NTR expression is increased in different models of HD, and the normalization of p75NTR expression was shown to be associated with the improve of behavioural alterations in HD (Brito et al., 2013, 2014; Miguez et al., 2015; Suelves et al., 2019). Overall, the imbalance in BDNF neurotrophic receptor signalling in HD takes part in the neuropathology and symptomatology (Figure 9).

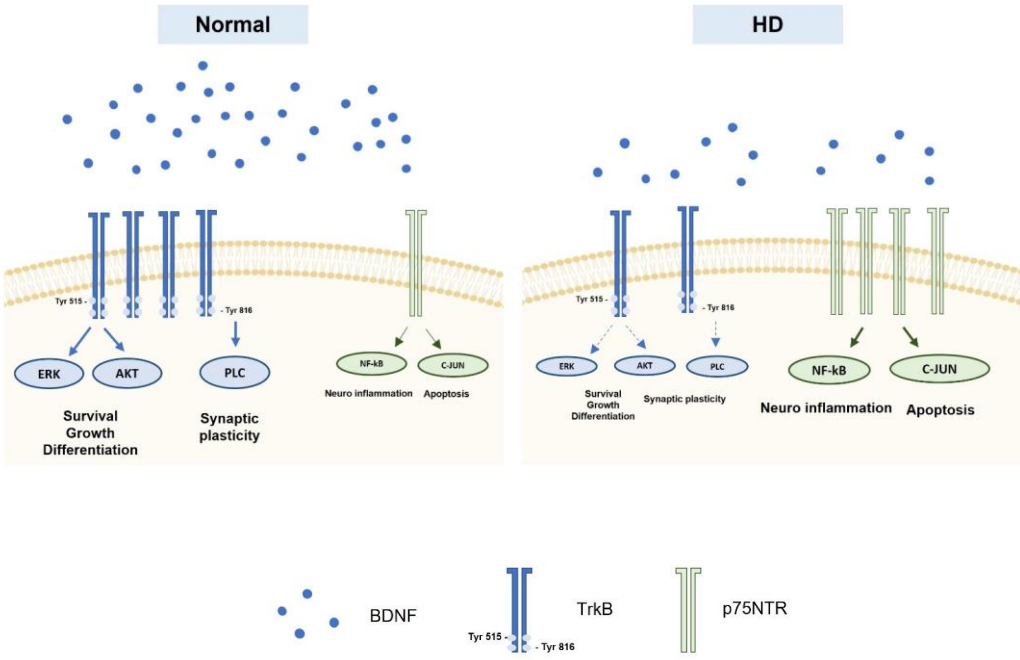


Figure 9: Mechanisms underlying neurotrophic dysfunction in HD. Schematic diagram showing the neurotrophic impairment described in HD. Under normal conditions, protein levels and activity of brain-derived neurotrophic factor (BDNF), tropomyosin receptor kinase (TrkB), and p75 neurotrophin receptor (p75NTR) are appropriate for the survival and functioning of the neuronal cells. When BDNF interacts with TrkB, it triggers activation of the extracellular signal-regulated kinase (ERK), Akt, and phospholipase C (PLC) pathways. In HD, BDNF protein levels are reduced because of mutant huntingtin-mediated transcriptional dysregulation and vesicular transport impairment of BDNF. Moreover, there is an imbalance in BDNF receptor levels: TrkB receptor protein levels, which enhance neuroprotective pathways, are decreased, whereas protein levels of p75NTR, which enhances degenerative signalling, are increased. Remarkably, TrkB signalling is also impaired independently of the TrkB protein levels.

Because molecular alterations and neuronal dysfunction precede neurodegeneration in HD, it has been proposed that pharmacological compounds that can stop these molecular aberrations and restore neuronal function could be potential therapeutic agents for HD.

2. Activation of PACAP receptors as a therapeutic approach in Huntington's disease

Pituitary adenylate-cyclase activating polypeptide (PACAP) is a neuropeptide member of the VIP-secretin-GHRH-glucagon superfamily (Miyata et al., 1989) considered a potential therapeutic agent for neurodegenerative diseases as it can protect neuronal and glial cells through the activation of its receptors.

2.1. PACAP and its receptors in the CNS

PACAP was discovered and isolated from an ovine hypothalamus extract in 1989 by Akira Arimura et al. based on its ability to stimulate adenylyl cyclase (AC) activity in pituitary cells (Miyata et al., 1989). PACAP exists in two isoforms, PACAP38 and PACAP27, which differ by 11 amino acid residues at the C-terminus (Fig 10, A). Both isoforms are derived from proteolysis of the PACAP precursor and are bioactive; however, PACAP38 is more than 100-fold abundant than PACAP27 in neuronal tissue

(Miyata et al., 1989, 1990). PACAP can cross the blood-brain barrier (BBB) in both directions. The influx of PACAP from blood circulation to the brain is the highest among the secretin/glucagon/GHRH superfamily. The crossing of PACAP38 trough the BBB is mediated by the specific peptide transporter system 6 whereas PACAP27 enters the CNS by passive diffusion (Banks et al., 1993). In humans, PACAP is encoded by the ADCYAP1 gene, located on chromosome 18p11 (Hosoya et al., 1992). The primary structure of PACAP38 is identical in humans, ovine, rats, and mice, indicating that it is highly conserved in mammals (Okazaki et al., 1995). Importantly, the sequence of human PACAP shares nearly 70% amino acid sequence identity with vasoactive intestinal polypeptide (VIP) (Figure 10, A). However, the potency of PACAP to activate the AC in neurons is 1,000 to 10,000 times greater than VIP (Arimura et al., 1994). The homology of the amino acid sequences of PACAP and VIP makes them share three class G protein-coupled receptors (GPCRs): PAC1 receptor (PAC1R), VPAC1 receptor (VPAC1R), and VPAC2 receptor (VPAC2R). Importantly, PACAP binds with high affinity to all three receptors, while VIP binds with high affinity to VPAC1R and VPAC2R and has a 1,000-fold lower affinity for PAC1R than PACAP (Figure 10, B).

(A)

	5 10 15 20 25 30 35
PACAP38	H S D G I F T D S Y S R Y R K Q M A V K K Y L A A V L G K R Y K Q R V K N K - NH_2
PACAP27	- NH_2
VIP	- - - A V - - - N T L - - - - - - - - - - N S I - N - NH_2

(B)

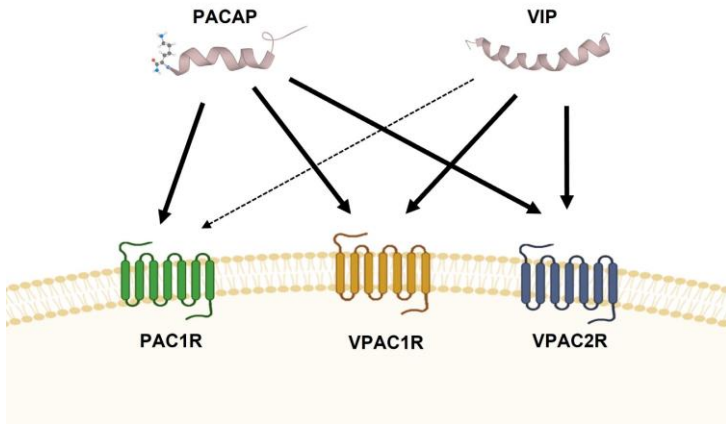


Figure 10: Aminoacidic nature of PACAP and VIP and their affinity for their receptors. (A) Schematic representation of amino acid sequences of PACAP38, PACAP27, and VIP. - Amino acids identical to PACAP. A modified version of the Vaudry et al. (2009). **(B)** Schematic representation of PACAP and VIP receptor affinity. PACAP binds with high affinity to PAC1R, VPAC1R, and VPAC2R, whereas VIP binds with high affinity to VPAC1R and VPAC2R, showing very low affinity for PAC1R.

2.1.1. Distribution of PACAP and its receptors in the CNS

Exploration of the expression of PACAP and its receptors in the brain has typically been performed using immunohistochemistry and *in situ* hybridization. In both humans and rodents, the highest concentrations of PACAP are found in the hypothalamus (Köves et al., 1991; Ghatei et al., 1993; Palkovits et al., 1995). However different studies with human, rat and mouse brains reveal that significant amounts of PACAP are also found in cerebral cortex, the hippocampus, the thalamus, the striatum, the nucleus accumbens, the substantia nigra, the locus coeruleus, and the pineal gland (Köves et al., 1991; Ghatei et al., 1993; Palkovits et al., 1995; Condro et al., 2016). Interestingly, the regional distribution of PACAP usually differs from that of VIP, indicating that their roles in the CNS are not identical (Besson et al., 1979; Ogawa et al., 1985). Additionally, the distribution of PACAP does not always parallel the localization of its receptors (Figure 11).

The relative distribution of PACAP receptors has been studied principally in the rat brain. Higher concentrations of PAC1R occur in many brain structures including the olfactory bulb, cerebral cortex, septum, amygdala, hippocampus, thalamus, hypothalamus, and substantia nigra (Cauvin et al., 1991; Masuo et al., 1991, 1992; Basille et al., 1994; Hou et al., 1994). Significative PAC1R abundance is also present in cerebellum (Basille et al., 1994) and pons (Cauvin et al., 1991; Masuo et al., 1992). In contrast, VPACR are mainly located in the olfactory bulb, cerebral cortex, DG of the hippocampus, pineal gland, and thalamus (Besson et al., 1984; Martin et al., 1987; Vertongen et al., 1998). According to mouse brain RNA-Seq dataset from the Human Protein Atlas (HPA), PAC1R is the most predominant receptor in the mouse brain, since its expression is much higher than that of VPACRs, and the hypothalamus, basal ganglia, and olfactory bulb are the three regions with higher levels (Sjöstedt et al., 2020). Importantly, results from the Allen Mouse Brain Atlas obtained by *in situ* hybridization also indicate that these areas have high expression of PAC1R but also show that the DG of hippocampal formation is the region with the highest expression of PAC1R (Allen Institute for Brain Science, 2004). On the other hand, VPAC1R is highly expressed in the cerebral cortex, hippocampal formation, amygdala, and the basal ganglia (Sjöstedt et al., 2020). Finally, higher levels of VPAC2R are found in the olfactory bulb (Sjöstedt et al., 2020) (Figure 11).

Remarkably, PACAP receptors are expressed in brain regions of special interest in the context of HD. All three receptors are present in the cerebral cortex. While VPAC1R and VPAC2R are localized in layers II-VI, higher levels of PAC1R are found in layers II, III, V, and VI and white matter (Joo et al., 2004; Zhang et al., 2021). In the hippocampus, pyramidal cells of CA1, CA2 and CA3, and granule cells of the DG express all receptors, being PAC1R especially expressed in granule cells from DG (Joo et al., 2004; Vaudry et al., 2009). Dorsal striatum and pallidum are also regions rich in all PACAP receptors (Joo et al., 2004; Vaudry et al., 2009). Finally, in the thalamus, subthalamic nucleus,

and in the substantia nigra, all three receptors are also present (Martin et al., 1987; Masuo et al., 1991; Joo et al., 2004).

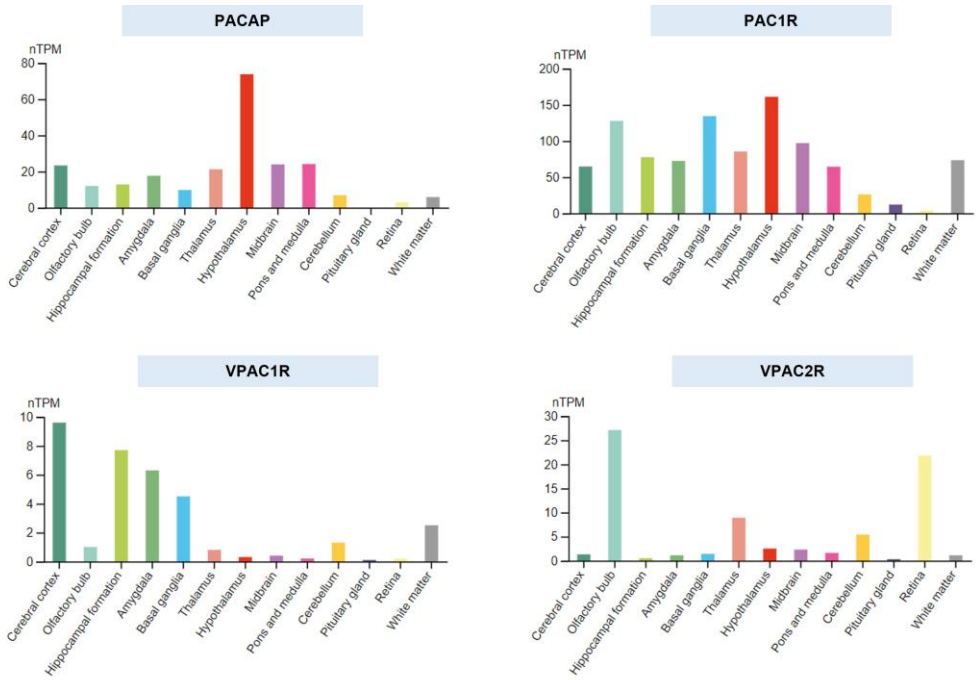


Figure 11: Expression of PACAP and its receptors thorough the mouse brain. Human Protein Atlas (HPA) RNA-seq tissue data is reported as mean nTPM (normalized expression) for each of the brain regions analyzed in mouse. The detailed nTPM values at the individual sample level and the detailed description of HPA RNA-seq assay can be found in (The Human Protein Atlas, n.d.). Image extracted from (Sjöstedt et al., 2020).

2.1.2. Function of PACAP and its receptors in the CNS

The widespread distribution of PACAP and its receptors in the brain has led to many hypotheses regarding the physiological functions of these receptors. The creation of knockout mutant mice of VIP, PACAP, PAC1R, VPAC1R and VPAC2R has allowed experimental validation of the number of physiological functions of PACAP receptors.

Related to embryonic brain, PACAP receptors are implicated in CNS development and maturation by promoting neural tube patterning and neurogenesis. VIP and PACAP

secreted from maternal and embryonic tissues interact with their natural receptors expressed differently in various developing structures and stimulate cell generation, migration, differentiation, synaptogenesis, and myelinization (Maduna and Lelievre, 2016).

In the adult brain, PACAP acts as a neurotrophic factor, neurotransmitter, neuromodulator, and neurohormone in different brain areas.

It is known that PACAP receptors expressed in pineal gland and suprachiasmatic nucleus play important roles in the rhythmicity of melatonin production and the control of circadian rhythms (Cagampang et al., 1998; Shen et al., 2000; Patton et al., 2020; Stangerup and Hannibal, 2020). On the other hand, PACAP-PAC1R signaling regulates the hypothalamic-pituitary-adrenal axial stress response. It has been found that PACAP modulates the function of the bed nucleus of the stria terminalis (BNST) and increases anxiety-like behavior (Hammack et al., 2003). Accordingly, PACAP and PAC1R are upregulated following chronic stress and PAC1R- deficient mice exhibit reduced anxiety behavior (Otto et al., 2001; Hammack et al., 2003). In addition, perturbations in the PACAP-PAC1R pathway have been suggested to be involved in abnormal stress responses underlying posttraumatic stress disorder (Ressler et al., 2011). Growing body of evidence suggests that PACAP also regulates food intake primarily via PAC1R, as PACAP-mediated suppression of appetite is absent in mice lacking PAC1R (Vu et al., 2015; Sureshkumar et al., 2022).

Importantly, studies on PAC1R knockout mice indicate that PACAP and PAC1R are involved in both LTP and hippocampus-dependent associative learning (Otto et al., 2001; Matsuyama et al., 2003). Cerebral administration of a VIP receptor antagonist in adult rats resulted in deficits in the Morris water maze test, indicating that VPACR are also implicated in learning and memory processes (Glowa et al., 1992). In fact, VPAC1R and VPAC2R are considered key modulators of hippocampal synaptic transmission,

pyramidal cell excitability, and long-term depression (LTD) (Cunha-Reis et al., 2006; Caulino-Rocha et al., 2022).

Finally, there is clear evidence that endogenous PACAP exerts neuroprotective effects against various types of injury. The neurotrophic role of PACAP is activated in several pathological conditions such as nerve transection, neuronal inflammation, stroke, and traumatic brain injury (Somogyvari-Vigh and Reglodi, 2004; Reglodi et al., 2011; Shioda and Nakamachi, 2015). In accordance, mice lacking PACAP show an increased vulnerability to cerebral ischemia (Ohtaki et al., 2006), traumatic spinal cord injury (Tsuchikawa et al., 2012), and retinal ischemia (Szabadfi et al., 2012). Unsurprisingly, exogenous PACAP has been proven to be a potent neuroprotective agent in several neuronal injury models, both *in vitro* and *in vivo* (Somogyvari-Vigh and Reglodi, 2004; Vaudry et al., 2009; Reglodi et al., 2011, 2017; Shioda and Nakamachi, 2015; Manavalan et al., 2017).

2.2. Neuroprotective signaling pathways activated by PACAP

As mentioned previously, PACAP family receptors belong to the superfamily of GPCRs, which initiate a wide variety of signalling pathways. All three receptors of PACAP family predominantly couple to the G_s protein, which promotes the activation of AC and production of 3',5'-cyclic adenosine monophosphate (cAMP) (Spongier et al., 1993; Sreedharan et al., 1994; Xia et al., 1997; Jóźwiak-Bębenista et al., 2007). cAMP acts as a second messenger in various signalling pathways and enhances the activation of the PKA (Skålhegg and Tasken, 2000; Waltereit and Weller, 2003). In contrast, PACAP receptors can couple to $G_{q/11}$, resulting in the activation of PLC, which cleaves phosphatidylinositol 4,5-bisphosphate to form diacylglycerol (DAG) and 1,4,5-trisphpshpate (IP3) (Spongier et al., 1993; Sreedharan et al., 1994; MacKenzie et al., 1996, 2001). DAG activates PKC, whereas IP3 stimulates the release of Ca^{2+} from the endoplasmic reticulum (Journot et al., 1994; Holighaus et al., 2011; Blechman and Levkowitz, 2013). VPAC1R and VPAC2R also elicit PLC by interacting with $G_{i/o}$ (van

Rampelbergh et al., 1997). Importantly, PACAP receptor-mediated activation of PKA and PKC can activate ERK signalling (Waltereit and Weller, 2003; Emery and Eiden, 2012; May et al., 2014; Georg et al., 2016). ERK belongs to the MAPK family and is an important effector of GPCRs that regulate cellular growth, division, differentiation, and apoptosis (Pelech and Sanghera, 1992; van Gastel et al., 2018). Additionally, PAC1R can activate endosomal PI3K/Akt signalling cascades through PKA to facilitate neuronal survival (Bhave and Hoffman, 2004; Miura et al., 2013).

The multiple protective effects of PACAP have been described to be facilitated through the activation of these pathways. Importantly, the neuroprotective effect of PACAP can be directed to neurons or mediated by glial cells, which also express PACAP family receptors (Joo et al., 2004; Shioda and Nakamachi, 2015; Linghai Kong et al., 2016; Karunia et al., 2021). Next, the principal neuroprotective signalling cascades and molecular mechanisms activated by PACAP receptors are summarized (Figure 12).

2.2.1. Molecular mechanisms underlying the anti-apoptotic effect of PACAP

One of the most described neuroprotective actions of PACAP is its anti-apoptotic effect. This capacity has been demonstrated against many neuronal insults, such as hydrogen peroxide, ethanol, and C2-ceramide, among others, and it is associated with different signaling pathways in different cell types (Vaudry et al., 2000, 2002; Falluel-Morel et al., 2004; Dejda et al., 2008) . In cultured granule neurons, PACAP prevents apoptotic cell death through the PKA- and PKC-dependent inhibition of caspase-3 (Vaudry et al., 2000). Moreover, a growing body of evidence suggests that ERK activation takes a role in this process by enhancing c-fos and B-cell lymphoma 2 (Bcl-2) expression, since in the mitochondria Bcl-2 blocks the cytochrome c release into the cytosol preventing caspase-3 activation (Vaudry et al., 1998; Falluel-Morel et al., 2004; Aubert et al., 2006; Pugh and Margiotta, 2006).

The ability of PACAP to inhibit caspase-3 activation via the CREB-Bcl-2 signaling pathway has also been described in retinal ganglion cells (Ye et al., 2019). Interestingly, another study on cerebellar granular cells demonstrated that PI3K/Akt activation also underlies the neuroprotective effect of PACAP against KCl-induced apoptosis (Bhave and Hoffman, 2004). In PC12 cells with β-amyloid- or rotenone-induced neurotoxicity, PACAP neuroprotective activities have also been demonstrated to be through the inhibition of caspase-3 with the implication of PKA and ERK pathways (Onoue et al., 2002a; Wang et al., 2005).

It has been suggested that PAC1R is the main effector of PACAP- induced antiapoptotic effects (Seaborn et al., 2011). However, more studies are needed to determinate the implication of all receptors in the PACAP -mediated anti-apoptotic effect.

2.2.2. Molecular mechanisms underlying neurotrophic actions of PACAP

Apart from inhibiting the apoptotic machinery, different findings point out that PACAP can promote neurotrophic signaling specifically through PAC1R. Several *in vitro* and *in vivo* studies have shown that PAC1R stimulation increases the transcription and expression of BDNF. In cultured rat cortical and hippocampal neurons, PACAP-PAC1R signaling induces the transcription of BDNF through the activation of the AC/PKA signaling pathway (Yaka et al., 2003; Dong et al., 2010). Additionally, PAC1R activation has been shown to enhance the phosphorylation of CREB and the expression of BDNF in various *in vitro* and *in vivo* neurodegenerative models(Rat et al., 2011; Brown et al., 2013; Cabezas-Llobet et al., 2018).

It has been seen that in hippocampal neurons PACAP can exert a trophic effect through the transactivation of TrkB receptor by PAC1R (Lee et al., 2002). In addition, it has been proposed that this crosstalk between TrkB receptors and PAC1R may involve the Src kinase family (Lee et al., 2002). Interestingly, PACAP-mediated activation of the Akt pro-survival pathway has also been suggested to be a consequence of TrkB activation (Lee et al., 2002).

2.2.3. Molecular mechanisms underlying modulatory actions of PACAP in synaptic plasticity

Interestingly, PACAP is also implicated in synaptic plasticity, as it can modulate the activity of NMDARs in the hippocampus (Liu and Madsen, 1997, 1998). Both PKA and PKC have been implicated in the PACAP-mediated enhancement of NMDAR, as well as Src family tyrosine kinase proteins. These effects on synaptic plasticity have been associated with PAC1R activation (Yaka et al., 2003; Macdonald et al., 2005). However, other studies demonstrate that VPACR are also implicated in the modulation of synaptic plasticity in a PKA-dependent matter, still the molecular mechanisms have not been deciphered yet (Cunha-Reis et al., 2006; Caulino-Rocha et al., 2022).

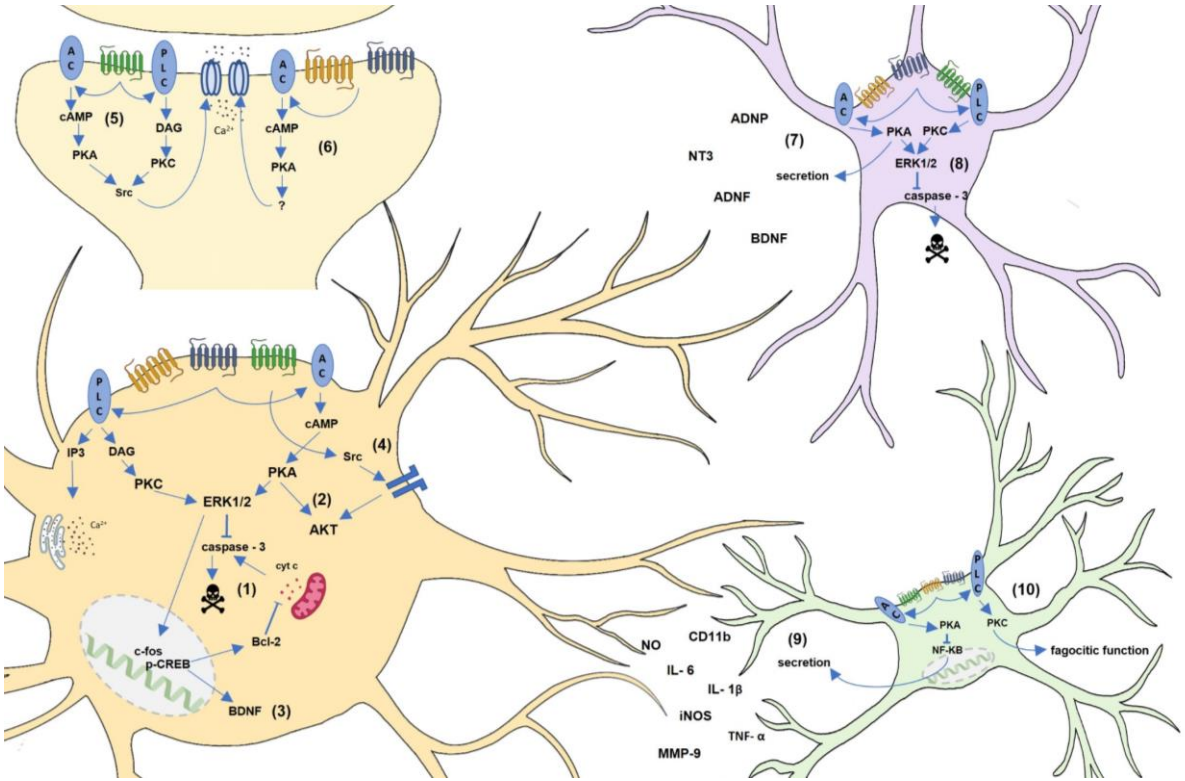
2.2.4. Molecular mechanisms underlying glial-mediated neuroprotective actions of PACAP

Numerous studies have demonstrated that the presence of PACAP receptors in glial cells allows them to mediate the effects of PACAP in the brain.

In astrocytes, PACAP induces the secretion of neurotrophic and neuroprotective factors, such as activity-dependent neuroprotective protein (ADNP) (Gozes et al., 2004), activity-dependent neurotrophic factor (ADNF) (Brenneman et al., 1997), neurotrophin-3 (NT3) (Brenneman et al., 1997), and BDNF (Passemard et al., 2011). The specific involvement of PACAP receptors in astrocyte-mediated neuroprotection has been poorly studied; however, some results suggest that VPACR play a key role in this action (Brenneman et al., 2003; Masmoudi-Kouki et al., 2007). In addition to this astrocyte-mediated neurotrophic action, PACAP can exert a potent protective effect against oxidative stress-induced apoptosis in astrocytes. Similar to what has been described in neurons, this anti-apoptotic activity is mediated through the PKA, PKC, and MAPK transduction pathways, and is associated with a decrease in caspase-3 activity (Masmoudi-Kouki et al., 2011) .Importantly, the glioprotective action of PACAP

against beta-amyloid peptide- and hydrogen peroxide-induced cell death has been demonstrated to be associated with PAC1R activation (Shieh et al., 2008; Masmoudi-Kouki et al., 2011) .

Finally, PACAP can modulate activated microglia, triggering anti-inflammatory effects (Karunia et al., 2021). It has been demonstrated that the activation of PACAP receptors can prevent the microglia-mediated secretion of pro-inflammatory factors such as iNOS, interleukin 1β (IL-1β), nitric oxide (NO), CD11b, matrix metallopeptidase 9 (MMP-9), interleukin 6 (IL-6), and tumor necrosis factor α (TNF- α) (Delgado and Ganea, 2003; Song et al., 2012; Broome et al., 2022). This action has been associated with the activation of the cAMP/PKA pathway and inhibition of nuclear translocation and DNA binding of the NF-κB (Delgado, 2002; Delgado et al., 2008). Interestingly, VPACR activation in microglia promotes microglial phagocytic activity through activation of the PKC signaling pathway, which has been demonstrated to be protective in the presence of fibrillar amyloid beta (Aβ) 42 (Song et al., 2012).



PAC1R
VPAC1R
VPAC2R
Phospholipase C
Adenylate cyclase
N-methyl-D-aspartate (NMDA)
TrkB receptor

IP3	1,4,5-trisphpshpate
DAG	diacylglycerol
PKC	protein kinase C
cAMP	3',5'-cyclic adenosinemonophosphate
PKA	protein kinase A
ERK 1/2	extracellular signal-regulated kinases 1/2
Bcl-2	B-cell lymphoma 2
NT3	neurotrphin-3
BDNF	brain derived neurotrophic factor
ADNF	activity- dependent neurotrophic factor
ADNP	activity- dependent neuroprotective
IL-6	interleukin 6
NO	nitric oxide
MMP-9	matrix metallopeptidase 9
TNF-α	tumor necrosis α
IL-1β	interleukin β
NF-KB	nuclear factor kappa-light-chain- enhancer of activated B cells

Figure 12: Schematic representation of molecular mechanisms underlying PACAP neuroprotective action. Because all PACAP receptors belong to the superfamily of G protein-coupled receptors, the beneficial effects of PACAP are associated with the activation of their related signalling pathways: AC/cAMP/PKA, PLC/PKC, ERK, and PI3K/Akt. **(1)** In neurons, PACAP prevents apoptotic cell death through PKA- and PKC-dependent inhibition of caspase-3 with the participation of ERK, which enhances the expression of c-fos and Bcl-2. In mitochondria, Bcl-2 blocks cytochrome c release into the cytosol, preventing caspase-3 activation. **(2)** Activation of PI3K/Akt through PKA also underlies the neuroprotective effect of PACAP against apoptosis. **(3)** The neurotrophic effect of PACAP is mediated by PAC1R stimulation, which promotes the expression of BDNF through activation of the AC/PKA signaling pathway and phosphorylation of CREB. **(4)** PACAP can promote transactivation of the TrkB receptor by PAC1R, involving the Src kinase family. **(5)** PACAP acts as a modulator of synaptic plasticity since PAC1R can control the activity of N-methyl-D-aspartate receptors (NMDAR) through PKA and PKC pathways, with the participation of Src family tyrosine kinase proteins. **(6)** VPACR are also implicated in the modulation of synaptic plasticity in a PKA-dependent manner; however, the molecular mechanisms have not been deciphered yet. **(7)** In astrocytes, PACAP induces the secretion of neurotrophic and neuroprotective factors, such as ADNP, ADNF, NT3, and BDNF with VPACR playing a key role in this process. **(8)** PACAP can be anti-apoptotic in astrocytes through PKA, PKC, and ERK transduction pathways that results in a decrease in caspase-3 activity. **(9)** PACAP receptors can prevent microglia-mediated secretion of pro-inflammatory factors, such as iNOS, IL-1β, NO, CD11b, MMP-9, IL-6, and TNF-α. This action has been associated with activation of the cAMP/PKA pathway and inhibition of nuclear translocation and DNA binding of NF-κB. **(10)** VPACR activation in microglia promotes microglial phagocytic activity through activation of the PKC signaling pathway.

2.3. Pharmacological use of PACAP in neurodegenerative diseases

Owing to its well-described neuroprotective action as well as its capacity to cross the BBB (Amin and Schytz, 2018), the therapeutic action of PACAP has been studied in multiple *in vitro* and *in vivo* models of neurodegenerative diseases.

In studies of Alzheimer's disease (AD), different *in vitro* investigations have demonstrated that PACAP protects against Aβ-mediated toxicity (Onoue et al., 2002a; Han et al., 2014). Additionally, long-term daily intranasal PACAP administration ameliorated cognitive deficits in an AD transgenic mouse model overexpressing amyloid precursor protein (APP). This improvement in cognitive function was associated with an increase in BDNF and PAC1R protein levels, enhancement of the non-amyloidogenic pathway of APP, and reduction of the inflammatory response (Rat et al., 2011). Interestingly, the capacity of PACAP to counteract cognitive decline has also been demonstrated in SAMP8 mice, another Aβ-expressing AD model (Nonaka et al., 2012).

In therapeutic studies of Parkinson's disease (PD), PACAP has been shown to be neuroprotective in different types of PD-cellular models induced by 6-hydroxydopamine (6-OHDA), 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, salsolinol, and paraquat neurotoxic agents (Takei et al., 1998; Chung et al., 2005; Wang et al., 2005; Brown et al., 2013; Lamine et al., 2016; Hajji et al., 2019). These findings are consistent with the results obtained in PD animal models. PACAP can improve motor and behavioral disturbances in 6-OHDA lesioned rats (Reglodi et al., 2004a, 2004b) as well as enhance the learning and memory deficits in an MPTP PD-induced mouse model (Deguil et al., 2009).

Overall, these studies indicate that PACAP, probably through PAC1R, activates different molecular mechanisms to protect and improve the functionality of neuronal cells, resulting in the enhancement of behavioral deficits associated with different

neurodegenerative diseases. Although PACAP is considered a potential therapeutic agent against neurodegenerative diseases for several years, its therapeutic applications have some limitations, as described in the section below.

2.3.1. The creation of PACAP analogues

The use of PACAP for the treatment of neurodegenerative diseases is hampered by different reasons. The first limitation is the metabolic instability of the peptide. The half-life of PACAP38 is estimated to be less than 2 minutes in mice (Zhu et al., 2003) and approximately 5 minutes in human plasma (Bourgault et al., 2008). This is because PACAP is rapidly metabolized mainly by dipeptidyl peptidase IV (DPP IV). DPP IV is a ubiquitous serine amino-terminal dipeptidase implicated in the degradation of several peptides and hormones, including PACAP (Zhu et al., 2003). The enzymatic action of DPP IV on PACAP38 and 27 leads to the cleavage of the two first amino acids and the formation of PACAP (3-38) and PACAP (3-27) fragments, respectively. DPP IV can also cleave PACAP (3-38) metabolite, resulting in the formation of PACAP (5-38) (Bourgault et al., 2008). Since the amino-terminal domain of PACAP is essential for receptor activation, cleavage by DPP IV can suppress the agonistic and biological activity of the peptide (Schäfer et al., 1999). Additionally, the products of resulting PACAP cleavage by DPP IV can act as PAC1R antagonists and counteract the beneficial effects of PACAP (Hou et al., 1994). Importantly, PACAP can also be cleaved by endopeptidases, which recognize two dibasic pairs (Arg^{14}-Lys^{15} and Lys^{20}-Lys^{21}), and carboxypeptidases, which act against the C-terminal segment (Bourgault et al., 2008).

The second limitation of the therapeutic use of PACAP is its lack of specificity. The physiological function of PACAP and the expression of its receptors are not restricted in the CNS, but also described in thyroid gland, gonads, adrenal gland, gastrointestinal tract, liver, pancreas, respiratory system, cardiovascular system, and immune cells (Vaudry et al., 2009). Different physiological responses related to the presence of PACAP receptors in these systems have been described following PACAP treatment.

For example, the intravenous infusion of PACAP38 for 20 minutes in humans significantly increases the heart rate, possibly because of its long-lasting vasodilator effect (Warren et al., 1992; Dorner et al., 1998; Birk et al., 2007). PACAP38 injection also causes a decrease in the partial pressure of carbon dioxide in healthy individuals and promotes an increase in ventilation in animal models (Naruse et al., 1993). Importantly, tachycardia, hypotension, and hyperventilation are thought to be mediated through VPACR (Bourgault et al., 2009a). First, because VPACR are largely distributed in peripheral tissues, including the airways, heart, kidneys, and vascular smooth muscles, while PAC1R is mainly localized in the CNS (Bourgault et al., 2009a). Second, because VIP, which has poor capacity to interact with PAC1R, causes similar biological activities after intravenous administration (Morice et al., 1983; Frase et al., 1987; Maxwell et al., 1990). Thus, considering that most of PACAP-related side effects are associated with VPACR activation, and that most of beneficial effects of PACAP are suggested to be mediated via PAC1R, there is special interest in identifying PAC1R-selective agonists to be used as effective therapeutic agents. Nowadays, the only selective PAC1R agonist available is maxadilan, a peptide isolated from the salivary glands of the sand flies (Lerner et al., 2007). Importantly, the therapeutic use of maxadilan is compromised by its structure which may be strongly immunogenic added to the fact that it is very difficult to synthesize (Bourgault et al., 2009a).

To overcome these two major limitations, chemical modification of the PACAP molecule has been proposed to enhance its stability and increase its selectivity for PAC1R. Different investigations have been performed to develop PACAP analogues with these properties. It has been found that the incorporation of an acetyl or hexanoyl group at the N-terminus of PACAP molecules completely suppresses the action of DPP IV, but only acetyl-PACAP derivatives maintain their full biological activity (Bourgault et al., 2008). In addition, substitutions on the cleavage sides of endopeptidases significantly prevent the proteolysis of PACAP. Finally, it has been proposed that the addition of an alkyl chain at the C-terminus can protect the molecule from the action

of carboxylases (Bourgault et al., 2008). Interestingly, addition of a propyl group at the end of the molecule did not alter the biological activity of PACAP (Bourgault et al., 2008). Related to PAC1R specificity, because the N-terminus is essential for the PAC1R-agonistic activity of PACAP, the substitution of one or more of the five initial residues of PACAP has been found to be a good approach to obtain PAC1R- preferring analogues (Ramos-Álvarez et al., 2015).

To date, the therapeutic potential of only two PACAP analogues has been studied in rodent models. On the one hand, Acetyl- [Ala15, Ala20] PACAP38 propylamine has been demonstrated to be a potent PAC1R agonist with a greater metabolic stability (Bourgault et al., 2008). Its half-life in the blood plasma is 25 minutes, and its affinity for PAC1R is four times greater (Bourgault et al., 2008). The functional effects of administrating this analogue have been poorly explored, and recent results showed that despite its antioxidative action, Acetyl- [Ala15, Ala20] PACAP38 propylamide did not improve the cognitive function nor increase BDNF expression as PACAP did in rats (Ladjimi et al., 2019). Interestingly, this analogue was also tested in a rat model of middle cerebral artery occlusion. In this case, Acetyl- [Ala15, Ala20] PACAP38 propylamide strongly reduced infarct volume and improved neurological impairment induced by stroke inhibiting apoptosis as well as modulating the inflammatory response (Dejda et al., 2011). On the other hand, Ac-[Phe(pI)6,Nle17] PACAP (1–27) is another analogue designed with a half-life of 2 hours in human plasma (Lamine et al., 2016). Interestingly, Ac-[Phe(pI) 6,Nle17] PACAP (1–27) was shown to protect against 1-methyl-4-phenylpyridinium (MPP+) toxicity in a cellular model of PD (Lamine et al., 2016). Moreover, its intravenously injection in MPTP-treated mice restored several molecular alterations in the substantia nigra and modulated the inflammatory response, provoking significantly less effects than PACAP38 in mean arterial blood pressure and no significant changes in heart rate (Lamine et al., 2016).

Importantly, in the context of HD, little was known about the therapeutic potential of PACAP before our laboratory began to explore it. The first study performed in a non-genetic model of HD obtained from a QA-induced striatal lesion showed that PACAP reduced striatal neuronal loss and attenuated behavioral disturbances (Tamás et al., 2006). Years later, our group demonstrated that PACAP counteracts cognitive decline in transgenic R6/1 mice. Importantly, these effects were associated with the increased expression of proteins related to synaptic plasticity, such as BDNF, as well as an increase in the expression of PAC1R (Cabezas-Llobet et al., 2018). Before the start of the present project, the ability of PACAP to improve motor deficits in R6/1 mice was well described by our group. However, the neuroprotective effects of PACAP against mHTT-induced toxicity were not fully explored. In addition, although the results indicated that PAC1R might be the main effector of neuroprotective effects in HD, its role was not entirely studied. Therefore, in the first part of this thesis, we focused on study the neuroprotective effect of PACAP against mHTT-induced toxicity and the role of PAC1R in this action. Because of the mentioned limitations of the pharmacological application of PACAP, we theorized that metabolically stable analogues of PACAP with higher affinity for PAC1R could represent novel therapeutic compounds for the treatment of HD. Thus, in the second part of this thesis we studied acetyl- [Ala15, Ala20] PACAP38 propylamine, which, although its neuroprotective action has been demonstrated in healthy Wistar rats and in a rat model of middle cerebral artery occlusion, it has never been tested in neurodegenerative diseases. On the other hand, we have also tested the Ac-[Phe(pI)6, Nle17] PACAP38, which the neuroprotective capacities of its form in PACAP27 have been demonstrated in a cellular and in a mouse model of PD as explained previously.

CHAPTER II: HYPOTHESIS AND OBJECTIVES

Hypothesis

Huntington's disease (HD) is a devastating neurodegenerative disorder with no effective treatment to prevent the natural progression of the disease. Motor and cognitive symptoms of HD are caused by progressive neuronal dysfunction and death in several brain areas, such as the striatum and hippocampus. Pituitary adenylate cyclase- activating polypeptide (PACAP) is a neuropeptide with described therapeutic capacities through the activation of its receptors, PAC1R, VPAC1R, and VPAC2R. Recently, our laboratory demonstrated that the administration of PACAP improves cognitive and motor phenotype in a transgenic mouse model of HD. However, the effects of PACAP on mHTT-induced cell toxicity, and the involvement of PACAP receptors in neuroprotection have not yet been explored. Importantly, the pharmacological application of PACAP is limited by its low bioavailability and lack of selectivity for its receptors. Because PAC1R is suggested to be the main effector of PACAP-induced neuroprotective effects, it could be a potential therapeutic target for HD. Therefore, metabolically stable analogues of PACAP displaying higher affinity for PAC1R could represent novel therapeutic compounds for the treatment of HD. Considering this background, the following hypothesis are proposed:

- PACAP can protect from mHTT-mediated toxicity mainly through PAC1R activation.
- Synthetic PACAP analogues showing higher affinity for PAC1R improve the symptomatology and neuropathology in a transgenic mouse model of HD.

Objectives

This thesis focuses on three principal aims:

1. **To study the capacity of PACAP to protect against mHTT-mediated toxicity and the involvement of PAC1R in striatal cells.**

1.1. To analyze the protein levels of PACAP receptors in STHdh cells.

1.2. To evaluate the neuroprotective effect of PACAP in striatal cells expressing mHTT.

1.3. To determine the contribution of PAC1R in the protective mechanisms enhanced by PACAP in STHdh cells.

2. **To study the effect of two PACAP analogues in the cognitive disturbances that occur in the R6/1 HD mouse model.**

2.1. To evaluate whether the intranasal administration of PACAP analogues improves learning and memory function.

2.2. To study whether PACAP analogues promote structural neuroplasticity in the hippocampus.

2.3. To decipher some of the molecular mechanisms initiated by PACAP analogues in the hippocampus.

3. **To assess the effect of two PACAP analogues in the motor discoordination that occurs in the R6/1 HD mouse model.**

3.1. To analyze whether the intranasal administration of PACAP analogues improves the motor function.

3.2. To study whether PACAP analogues prevent the striatal degeneration.

3.3. To identify some of the molecular mechanisms initiated by PACAP analogues in the striatum.

4. **To analyze whether the administration of two PACAP analogues in R6/1 HD mouse model reduces the mHTT aggregation in the hippocampus and striatum.**

CHAPTER III: METHODS

1. Cellular and animal models of HD

1.1. STHdh cell line

The two immortalized neuronal progenitor cell lines used in this work named STHdh were originally established at the laboratory of Dr. Marcy MacDonald (Harvard Medical School, Boston) (Trettel et al., 2000). STHdh cell lines were generated from striatal primordia of the HD knock-in mouse model, which carries a mutation inserted into the mouse huntingtin gene. Thus, STHdh cells stably express full-length wild-type (WT) HTT with 7 glutamines (STHdhQ7/Q7; Coriell Cat# CH00097, RRID:CVCL_M590) or full-length mHTT with 111 glutamines (STHdhQ111/Q111; Coriell Cat# CH00095, RRID:CVCL_M591) at endogenous levels (Trettel et al., 2000). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Saint Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, GE Healthcare, Chicago, IL, USA), 1% streptomycin-penicillin (Lonza, Basel, Switzerland), 2 mM L-glutamine (Lonza), and 1mM sodium pyruvate (Lonza). The cells were maintained in 100 mm dishes at 33ºC with 5% CO2, as previously described (Gines et al., 2003b).

1.2. Genetically modified R6/1 HD mouse model

R6/1 transgenic mice expressing exon-1 of mHTT containing 115 CAG (RRID: IMSR_JAX:006471) were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and maintained on a B6CBA background. Mice were genotyped using polymerase chain reaction (PCR) from ear biopsies as described previously (Mangiarini et al., 1996). Wild-type littermates were used as controls. All mice were male and housed together in groups of mixed genotypes, with ad libitum access to food and water. The colony room was maintained at 19–22°C and 40–60% humidity under a 12-hour light/dark cycle. Experiments were conducted in a blind-coded manner with respect to genotype and treatment, and data were recorded for analysis by the microchip mouse number. All

animal procedures were approved by the Animal Experimentation Ethics Committee of the University of Barcelona (274/18) and Generalitat de Catalunya (10101) in compliance with the Spanish RD 53/2013 and European 2010/63/UE regulations for the care and use of laboratory animals.

2. Peptides and pharmacological treatments

2.1. Peptides

The synthesis of PACAP, VIP and PACAP analogues was performed by the team of Dr. Vaudry (Normandie University, INSERM, Rouen; France). All peptides were produced using a solid-phase strategy combined with Fmoc chemistry methodology as previously described (Jolivel et al., 2009).

The analogues used in the present thesis are Acetyl- [Ala15, Ala20] PACAP38 propylamide and Ac-[Phe(pI)6, Nle17] PACAP38, referred as A1 and A2 respectively.

In the A1, the addition of the acetyl group suppresses the action of DPP IV, and the addition of propylamide protects the C terminal. These modifications increase its metabolic stability. On the other hand, the substitution of lysine residues 15 and 20 for alanine residues leads A1 to 4-fold increase of binding affinity for PAC1R as compared to PACAP38 and 2-fold increase in its potency to mobilize calcium (Bourgault et al., 2008).

In the A2, the addition of the acetyl group suppresses the action of DPP IV, and the substitution of methionine residue with norleucine isostere avoids peptide oxidation. These modifications increase its metabolic stability. In addition, the introduction of a bulky iodine atom on the para carbon of the aromatic ring of Phe6 enhances the affinity of the peptide for PAC1R (Bourgault et al., 2009b; Lamine et al., 2016).

2.2. Pharmacological treatments in STHdh cells

To analyze the protein levels of PACAP receptors, STHdhQ7/Q7 and STHdhQ111/Q111 cells were seeded in 60 mm dishes and treated at 70% confluence with phosphate-buffered saline (PBS) as vehicle or PACAP (10-7 M) for 24 hours.

To evaluate the capacity of PACAP and VIP to inhibit apoptosis, the cells were seeded in 60 mm dishes. When the cells reached 70% confluence, the medium was removed and fresh medium without FBS was added. The cells were then treated with PBS as vehicle, PACAP (10-7 M), or VIP (10-7 M) for 24 hours.

To study the activation of the ERK and Akt pathways after PACAP and VIP treatments, cells were seeded in 60 mm dishes and treated at 70% confluence with PBS as a vehicle, PACAP (10-7 M), or VIP (10-7 M) for 5, 15, and 30 minutes, and 1, 6, and 24 hours.

To determine the involvement of extracellular signal-regulated kinases 1/2 (ERK1/2) and Akt pathways in the inhibition of cleaved caspase-3, cells were seeded in in 60 mm dishes. When the cells reached 70% confluence, the medium was removed and fresh medium without FBS was added. The cells were exposed to PD98059 (25 µM), LY294002 (10 µM), or DMSO as a vehicle (all from Sigma-Aldrich) 30 minutes before treatment with PBS or PACAP (10-7 M) for 24 hours.

Finally, to study whether PACAP and VIP could induce the expression of neurotrophic and neuroprotective proteins, cells were seeded in 60 mm dishes and treated at 70% confluence with PBS or PACAP (10-7 M) for 6 and 24 hours.

The experimental design of *in vitro* study is summarized in Figure 13.

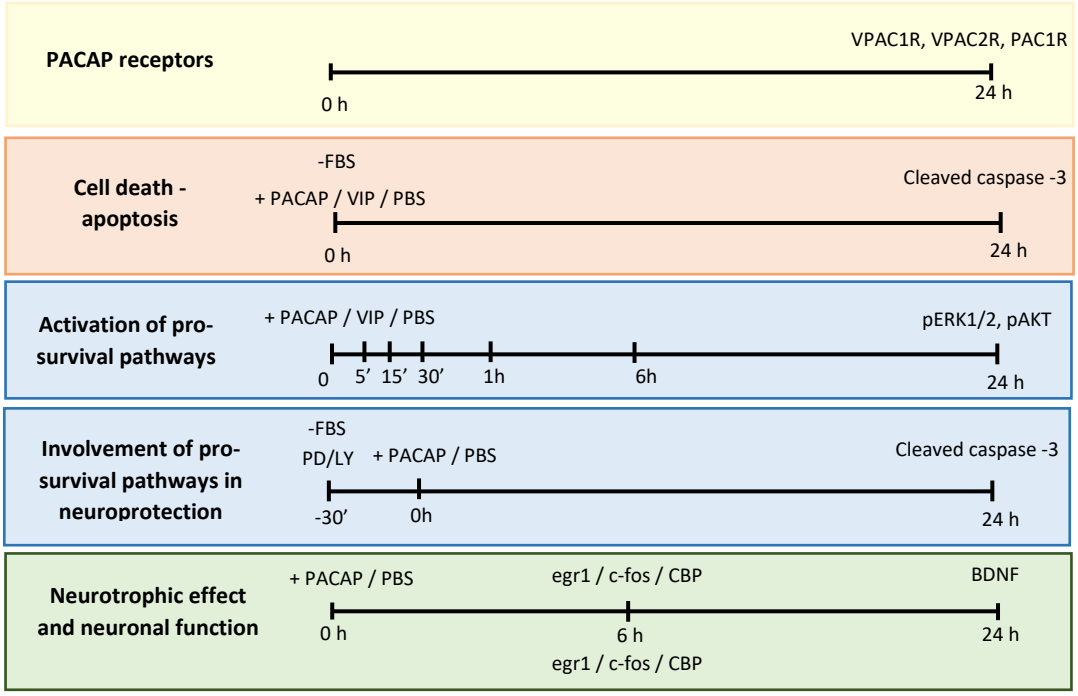


Figure 13: Experimental design performed in STHdh cellular model. STHdhQ7/Q7 and STHdhQ111/Q111 cells were treated at 70% confluence with PBS, PACAP (10^{-7} M) or VIP (10^{-7} M). To determine protein expression by western blotting, cells were treated for 5 minutes to 24 hours with peptides. To inhibit ERK and PI3K-Akt pathways, STHdhQ7/Q7 and STHdhQ111/Q111 cells were exposed to PD98059 (25 µM) or LY294002 (10 µM) 30 minutes prior to PACAP treatment.

2.3. Pharmacological treatments in animals

WT and R6/1 mice were treated intranasally with PBS as vehicle or with the analogue A1 or A2, at 30 µg/kg. The dose was determined based on previous studies (Cabezas-Llobet et al., 2018a; Solés-Tarrés et al., 2022). For cognitive assessment, treatment was initiated at 13-week-old, whereas for motor analysis, the first administration was made at 18-week-old. In both cases, administration was performed daily for 12 days, and behavioral testing was started on the 8th day of administration. During the testing days, the animals were treated 30 minutes before performing the behavioral tests. Last day of study, animals were euthanized by cervical dislocation 30 minutes after the

last administration. Experimental design of *in vivo* study is summarized in Figures 14 and 15.

3. Behavioral assessment

3.1. Cognitive assessment

T-MAZE test

The T-MAZE test is designed to assess spatial memory in mice and relies on rodents' innate preference for novelty. A wooden maze was used consisting of three arms; two of them were situated at 180º from each other, and the stem arm was positioned at 90º from the others. All arms were 45 cm long and 8 cm wide, with walls of 20 cm high. The T-MAZE protocol consisted of two sessions (training and testing) separated by an inter-trial interval (ITI) of 1 hour. The T-MAZE task was played at 8th day of treatment, and peptide administration was performed 30 minutes before the training session. The T-MAZE task was performed in a room with dim lighting (45-50 lux). Animals were tracked and recorded using SMART Junior software (Panlab). For the training session, mice were placed in the stem arm of the T-MAZE apparatus and allowed to explore the maze with only one accessible arm (old arm) for 10 minutes. Importantly, the arm chosen for the blockage was chosen randomly and alternated between mice. After one hour, in the testing session, the mice were placed again in the stem arm of the T-MAZE and allowed to explore the two arms for 5 minutes. Arm preference was determined as the time spent exploring each arm x 100/ time exploring both arms. Before each session, animals were habituated for at least 1 hour in the testing room (Figure 14).

Novel Object Recognition Test (NOLT):

Similar to T-MAZE, NOLT is designed to assess spatial memory in mice and relies on the innate preference of rodents for novelty. The NOLT protocol consisted of three

sessions performed on three consecutive days with an ITI of 24 hours: habituation (open field test), training, and testing. The first session took place at 9th day of treatment, and peptide administration was always performed 30 minutes before each session. All sessions were completed in an open-top arena (45 × 45 × 45 cm), presenting a visual cue in one of the walls, and placed inside a room with dim lighting (40 lx). Animals were tracked and recorded using SMART Junior software (Panlab). On the first day, animals were subjected to the open field test. Mice were habituated to the arena for 30 minutes while total distance travelled and the time spent in the central area were determined as a measure of locomotor activity and anxiety levels, respectively. On the second day of the training session, two identical objects were placed in the arena, and mice were allowed to explore them for 10 minutes. Then, 24 hours after the training session, one object was removed from its original location and placed diagonally to the other object (new location). Then, during the test, mice were allowed to explore them for 5 minutes. The object preference was measured as the time exploring each object x 100/ time exploring both objects. Before each session, animals were habituated for at least 1 hour in the testing room (Figure 14).

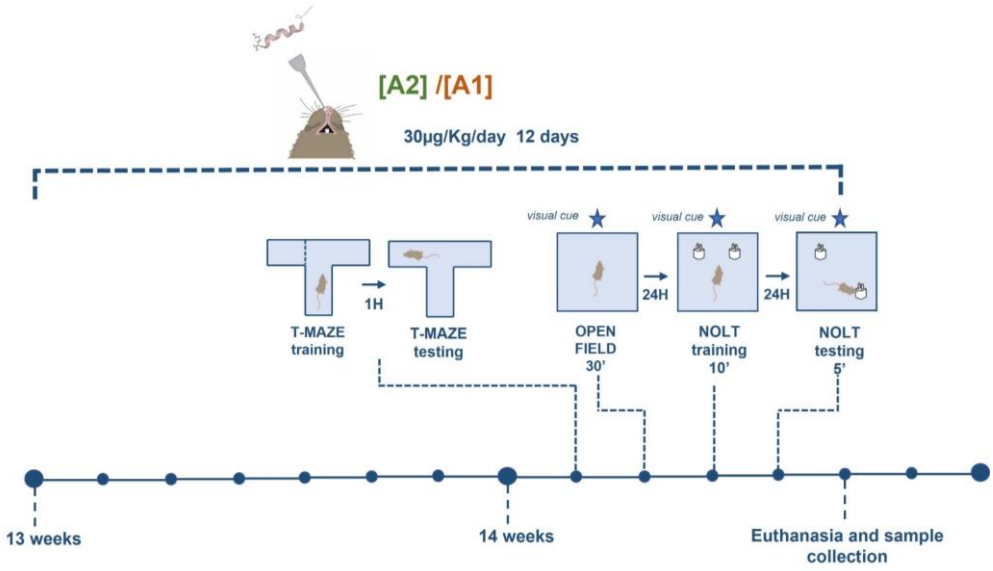


Figure 14: Experimental design to study the effects of A1 and A2 on cognitive function in WT and R6/1 mice. At the age of 13 weeks, mice were daily treated intranasally with PBS as vehicle, A1 or A2 (30 µg/kg/day) for 12 days. At the 8th day of treatment, cognitive function and started to be assessed for 4 days by the T-MAZE, and NOLT tests. On the 12th day of treatment, the animals were treated 30 minutes before sacrifice by cervical dislocation, and the samples were collected.

3.2. Motor assessment

Balance Beam

The balance of the animals was evaluated using the balance beam test, in which the ability of animals to cross a beam was measured. The beam consisted of 50-cm long square wooden steel with 1.3 cm face and divided into 5 cm frames. The beam was placed horizontally, 40 cm above the surface of the bench. At 8th day of treatment and 30 minutes after peptide administration, the animals were allowed to walk for 2 minutes along the beam, and the number of slips and the distance covered were measured. Before the test, the animals were habituated to the testing room for at least 1 hour (Figure 15).

Rotarod

Balance and motor coordination of animals were also evaluated using the rotarod apparatus. The animals were placed on a motorized cylinder (30 mm in diameter) at a fixed rotation speed. The number of falls in 60 seconds was recorded at speeds of 12, 16, and 24 rpm. Only trials in which the mice placed all four paws on the rod were considered valid and subsequently evaluated. Starting on the 9th day of treatment and 30 minutes after the administration, one speed per day was tested, from the lowest to the highest speed, for three consecutive days. Three weeks prior to the test, at the age of 16 weeks, the animals were subjected to six training sessions over 3 days at a speed of 12 rpm. During this learning phase, mice falling from the rod were returned, and the number of falls was recorded until the addition of the latencies to fall reached a total

time of 60 seconds per trial. Before the test, the animals were habituated to the testing room for at least 1 hour (Figure 15).

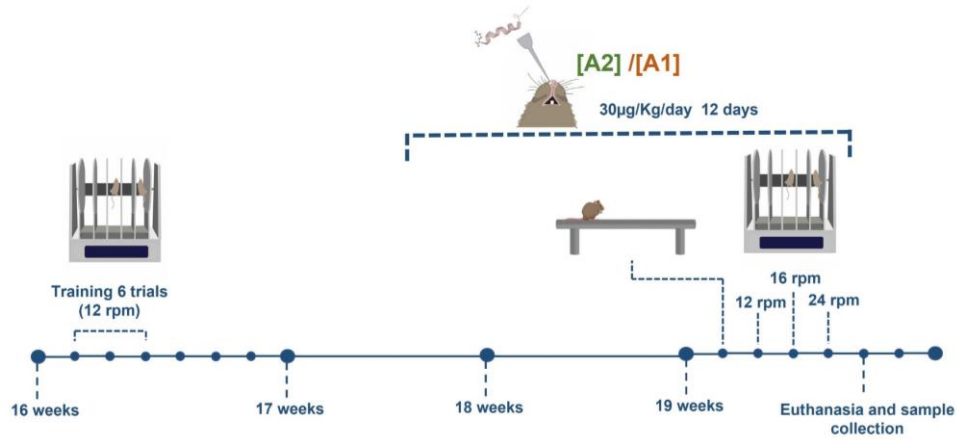


Figure 15: Experimental design to study the effects of A1 and A2 on motor function in WT and R6/1 mice. At the age of 18 weeks, the mice were treated daily intranasally with PBS as the vehicle and A1 or A2 (30 µg/kg/day) for 12 days. On the 8th day of treatment, motor analysis was performed for 4 days using the balance beam and rotarod tests. At the 12th day of treatment, animals were treated 30 minutes before to be sacrificed by cervical dislocation and the sample were collected.

4. Tissue preparation, histological staining, and imaging analysis

4.1. Tissue preparation and immunofluorescence

Mice were euthanized by cervical dislocation and left hemispheres were removed and fixed for 72 h in paraformaldehyde solution (4% in a phosphate buffer 0.1M). Then, serial coronal sections of 40 µm were obtained using a Leica Vibratome (Leica VT1000S). Free-floating sections were washed for 5 minutes 3 times in PBS, treated with NH_4Cl 50 mM for 30 minutes, and washed again 5 minutes 3 times with PBS. Floating sections were blocked and permeabilized with an incubation of 1 hour at room temperature with blocking/permeabilizing buffer: PBS containing 0.3 % Triton X-100

(PBS-T), 0.2% sodium azide, 2% bovine serum albumin (BSA, Sigma Aldrich), and 3% Normal Goat Serum (NGS; Pierce Biotechnology, Rockford, IL, USA). Then, slices were incubated overnight at 4°C with the corresponding primary antibodies diluted in blocking/permeabilizing buffer (Table 1). Then, sections were washed for 5 minutes 3 times with PBST-T and incubated 2 hours at room temperature with corresponding fluorescent secondary antibody diluted in blocking/permeabilizing buffer (Table 2). After 2 washes of 5 minutes with PBS, slices were incubated 10 minutes at room temperature with 4',6-diamidino-2-phenylindole (DAPI; 1:50000; Invitrogen, Carlsbad, CA, USA) for nuclear staining and washed again 10 minutes with PBS before being mounted with Mowiol (Merck, Darmstadt, Germany) on silane-coated slides. Immunofluorescence specificity was confirmed in slices without primary antibody. Sections were analyzed using a two-photon confocal microscope (Nikon Ti Eclipse).

Table 1: Primary antibodies used for immunofluorescence. A list of primary antibodies is provided, as well as their source and the dilution that was used.

ANTIGEN	HOST SPECIE	DILUTION	SOURCE	RRID
DARPP-32	Mouse	1/800	BD Biosciences 611520	AB_398980
Huntingtin Protein, clone EM48	Mouse	1/150	Millipore MAB5374	AB_177645
PSD-95	Mouse	1/300	Thermo Fisher Scientific MA1-046	AB_2092361
Synaptophysin	Rabbit	1/300	Millipore AB9272	AB_570874
Phospho-CREB (Ser 133)	Rabbit	1/600	Millipore 06-519	AB_310153

Table 2: Secondary antibodies used for immunofluorescence. A list of secondary antibodies is provided, as well as their source and the dilution that was used.

SECONDARY ANTIBODY	HOST SPECIE	DILUTION	SOURCE	RRID
Alexa Fluor 488 anti-rabbit	Donkey	1/300	Jackson ImmunoReserch Labs 711-545-152	AB_2313584
Alexa Fluor 488 anti-mouse	Donkey	1/200	Jackson ImmunoResearch Labs 715-545-150	AB_2340846
Alexa Fluor 647 anti-mouse	Goat	1/300	Thermo Fisher Scientific A21236	AB_2535805

4.2. Confocal imaging analysis

All fluorescently stained coronal sections were examined blinded to genotype and treatment, from at least five mice per group and using two-photon confocal microscope (Nikon Ti Eclipse) at 1,024 x 1,024-pixel resolution. Then, images were analyzed with ImageJ software (NIH, Bethesda, Montgomery,AL, USA).

DARPP-32 was imaged using the 40x objective with an additional electronic zoom of 1,996. At least six 40-µm slices containing dorsolateral striatum were analyzed per mouse and a representative image was obtained from each slice. Number of DARPP-32-positive neurons and DAPI-stained nuclei was obtained using the cell counter tool of ImageJ. Then, using the same software, the most centric neuron was manually delimitated, and the area and the mean intensity of DARPP-32 immunoreactivity was calculated using the Image J software.

In EM48- stained slices confocal *z*-stacks were taken every 1 µm using the 60x oil immersion objective with an additional electronic zoom of 1,996. At least four slices containing dorsolateral striatum and three slices containing dorsal hippocampus were analyzed per mouse. Representative images of the dorsolateral striatum and the hippocampal CA1, CA3, and DG were obtained from each slice. Number and volume of mHTT aggregates were obtained using the 3D-object counter tool of ImageJ. The volume analyzed was determined by multiplying the area of images by the height (number of confocal z-stacks of 1 µm analyzed). Then total number of mHTT aggregates was divided by the volume analyzed to obtain the number of mHTT aggregates per mm^3. Finally, the number and volume of aggregates was expressed as a percentage respect to R6/1-VEH mice.

Synaptophysin/PSD-95-stained slices were imaged using the 60x oil immersion objective with an additional electronic zoom of 5. At least three 40-µm slices containing dorsal hippocampus were analyzed per mouse. Two representative images of the CA1 and CA3- *stratum radiatum* and of the DG- *stratum moleculare* were obtained from each slice. The number of individual synaptic puncta (pre- and post-synaptic) was counted per field using the optimized threshold protocols selected within the ImageJ. Then, the software identified the co-localization of synaptic puncta as the overlap of 1 pixel or more.

p-CREB was imaged using the 60x oil immersion objective. At least two slices containing dorsal hippocampus were analyzed per mouse. A representative image of the CA1, CA3 and, DG was obtained from each slice. The intensity of p-CREB immunoreactivity was quantified applying a threshold appropriate to select individual neuronal cells within the ImageJ. Then, a mask was created to measure the mean staining intensity of the cells.

4.3. Golgi staining and spine analysis

After euthanasia, fresh brain hemispheres were processed following the Golgi-Cox method. Basically, mouse brain hemispheres were incubated in the dark for 23 days in filtered dye solution (10 g l^{-1} $K_2Cr_2O_7$, 10 g l^{-1} $HgCl_2$ and 8 g l^{-1} K_2CrO_4). The tissue was then washed for 2 minutes 3 times in deionized water and 30 min in 90% EtOH (v/v). Then, coronal sections of 200 µm were cut in 70% EtOH using a Leica Vibratome (Leica VT1000S). Brain slices were washed in deionized water for 5 minutes before being reduced in 16 % ammonia solution for 1 hour. After a wash of 2 minutes in deionized water, slices were fixed in 10 g l^{-1} $Na_2S_2O_3$ for 7 min. Then, slices were washed 2 minutes in deionized water and mounted on super-frosted coverslips. Next, slices were gradually dehydrated for 3 minutes in 50, 70, 80 and 100% EtOH solutions. Then, they were incubated for 5 minutes 2 times in a 2:1 isopropanol:EtOH solution, 5 minutes in pure isopropanol and finally 5 minutes 2 times in xylol before being mounted with DPX (Merck, Darmstadt, German).

Bright-field images of Golgi-impregnated dendrites in the *stratum radiatum* from hippocampal CA1 pyramidal neurons were captured with a Nikon DXM 1200F digital camera attached to a Nikon Eclipse E600 light microscope using the × 63 oil immersion objective and an additional electronic zoom of 1.6. Only fully impregnated pyramidal neurons with their soma entirely within the thickness of the section were used. Image *z*-stacks were taken every 0.2 µm. The total number of spines was obtained using the cell counter tool in the ImageJ software. Dendritic segments were traced, and spine density were quantified in at least 60 dendrites per group from at least five mice per group. A maximum of 2 dendrites per neuron were analyzed. Spine density analysis was performed blinded to genotype and treatment.

4.4. Cresyl violet staining and striatal volume analysis

Mice were euthanized by cervical dislocation and left hemispheres were removed and fixed for 72 h in paraformaldehyde solution (4% in a phosphate buffer 0.1M). Then, serial coronal sections of 40 µm were obtained using a Leica Vibratome (Leica VT1000S). Then, slices spaced 320 µm and covering the entire rostrocaudal extent of striatum, were selected and mounted on super-frosted coverslips. Slices were stained for 45 minutes in 0.1% Cresyl Violet (Nissl stain) and then gradually dehydrated for 5 minutes in 70 and 90% EtOH solutions and 5 minutes 2 times in a 100% EtOH solution. Then, they were incubated 5 minutes 2 times in xylol before being mounted with DPX (Merck, Darmstadt, German). Bright-field images of Nissl-stained brain were captured with a Nikon DXM 1200F digital camera attached to a Nikon Eclipse E600 light microscope using the × 2.5 objective. Then, striatal area was delimitated in each slice using the ImageJ software and striatal volume estimations were performed following the Cavalieri method. The analysis was performed blinded to genotype and treatment and from at least five mice per group.

5. Protein extraction and western blotting

In cell cultures, STHdhQ7/Q7 and STHdhQ111/Q111 cells treated with vehicle or with peptides were homogenized with ice-cold lysis buffer 1% Triton X-100, 10% glycerol, 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 150 mM NaCl, and 5µM $ZnCl_2$ supplemented with protease and phosphatase inhibitors (2 mM phenylmethylsulphonyl fluoride, 1 µg/µL aprotinin, 1 µg/µL leupeptin, 2 mM Na_3VO_4) (all from Sigma-Aldrich). Then, samples were centrifuged at 10000 rpm for 15 minutes at 4ºC, and supernatants were collected. In mice, after the brain removal, hippocampus, striatum, and motor cortex were dissected out and maintained at 80ºC. Then, all areas were homogenized with the same ice-cold lysis buffer described above and sonicated. Then, samples were then centrifuged at 15000 rpm for 15 minutes at 4ºC, and supernatants were collected. In

all cases, protein concentration was determined using the Dc protein assay kit (Bio-Rad Laboratories). Then, equal amounts of protein extracts (10-30 µg) were mixed with NuPAGE® sample reducing agent (ThermoFisher Scientific, MA, USA) and NuPAGE® lithium dodecyl sulfate sample buffer and heated for 10 minutes at 70 ◦C. Proteins were separated in polyacrylamide gels (SDS-PAGE) at different polyacrylamide concentrations and transferred to nitrocellulose membranes (ThermoFisher Scientific, MA, USA).

Blots were blocked in 5% non-fat powdered milk or 5% bovine serum albumin (BSA) Tris-buffered saline buffer containing 0.1% of Tween-20 (TBS-T, Sigma-Aldrich) for 1'5-2 hours at room temperature. The membranes were then incubated overnight at 4ºC with the corresponding primary antibodies diluted in blocking buffer (Table 3). Loading control was performed by reprobing the membranes with anti-α-tubulin or anti-β-actin primary antibodies depending on the molecular weight of the protein of interest. Membranes were washed in TBS-T and incubated with corresponding horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature (Table 4). Immunoreactive bands were detected using Immobilon Western Chemiluminiscent HRP Substrate (Millipore Corporation, Billerica, USA) in a Bio-Rad ChemiDoc™ MP Imaging System (Bio-Rad Laboratories). Bands corresponding to the molecular weight of each protein were quantified using Image Lab Software (Bio-Rad Laboratories). Then, protein expression of each subject was normalized to its own actin or tubulin levels.

Table 3: Primary antibodies used for western blot. A list of primary antibodies is provided, as well as their source, and blocking buffer and dilution used.

ANTIGEN	HOST SPECIE	DILUTION	BLOCKING BUFFER	SOURCE	RRID
PAC1R	Mouse	1/800	5% milk	Santa Cruz Biotechnology sc-100315	AB_1126992
VPAC1R	Rabbit	1/1000	5% milk	Abcam ab138260	AB_2935799
VPAC2R	Rabbit	1/1000	5% milk	Abcam ab28624	AB_778889
BDNF	Rabbit	1/500	5% milk	Santa Cruz Biotechnology sc-546	AB_630940
BDNF	Mouse	1/1000	5% milk	Icosagen 327-100	AB_2927780
CBP	Rabbit	1/500	5% milk	Santa Cruz Biotechnology sc-369	AB_631006
c-fos	Rabbit	1/500	5% milk	Santa Cruz Biotechnology sc-52	AB_2106783
Egr-1	Rabbit	1/1000	5% milk	Cell Signaling Technology 4154	AB_2097035
Cleaved-caspase 3	Rabbit	1/500	5% milk	Cell Signaling Technology 9661	AB_2341188
Caspase-3	Rabbit	1/1000	5% milk	Cell Signaling Technology 9662	AB_331439
phospho-ERK 1/2 (Thr202/Tyr204)	Rabbit	1/1000	5% milk	Cell Signaling Technology 9101	AB_331646
ERK 1/2	Mouse	1/1000	5% milk	BD Biosciences 610123	AB_397529
phospho- Akt (Ser473)	Rabbit	1/1000	5% milk	Cell Signaling Technology 9271	AB_329825
Akt	Mouse	1/1000	5% milk	BD Biosciences 610876	AB_398193
Trk B	Mouse	1/1000	5% BSA	BD Biosciences 610102	AB_397508
α-tubulin	Mouse	1/50000	5% milk	Sigma-Aldrich T9026	AB_477593

ANTIGEN	HOST SPECIE	DILUTION	BLOCKING BUFFER	SOURCE	RRID
β-actin	Mouse	1/1000	5% milk	Santa Cruz Biotechnology sc-47778	AB_626632
phospho-NMDAR2A (Y1325)	Rabbit	1/1000	5% BSA	Abcam ab16646	AB_2112289
phospho-NMDAR2A (Y1246)	Rabbit	1/1000	5% BSA	Cell Signaling Technology 4206	AB_2112292
NMDAR2A	Rabbit	1/1000	5% BSA	Cell Signaling Technology 4205	AB_2112295
phospho-NMDAR2B (Y1472)	Rabbit	1/1000	5% milk	Cell Signaling Technology 4208	AB_1549657
NMDAR2B	Rabbit	1/1000	5% milk	Cell Signaling Technology 4207	AB_1264223
p75NTR	Rabbit	1/1000	5% milk	Proteintech 55014-1-AP	AB_10858484

Table 4: Secondary antibodies used for western blot. A list of secondary antibodies is provided, as well as their source and dilution used.

SECONDARY ANTIBODY	DILUTION	SOURCE	RRID
Anti-Mouse	1/2000	Promega, Madison, USA W402B	AB_430834
Anti-Rabbit	1/2000	Promega, Madison, USA W401B	AB_430833

6. Statistical analysis

Normal distribution was tested using Anderson-Darling, Shapiro–Wilk, Kolmogorov–Smirnov, and d'Agostino-Pearson omibus normality tests. If one of these tests passed, a normal distribution was assumed. We used the Brown-Forsythe test to determine whether the variances in the comparison groups were equal. We used different tests to determine how one factor (treatment) influenced the response variable. For parametric data, we used unpaired two-sided Student's t-test to compare two groups and one-way ANOVA with Dunnett's or Tukey's post-hoc tests for multiple comparisons. When variances were unequal, Welch's t-test for comparing two groups and Welch's ANOVA test with Dunnett's or Thamane's T2 as a post hoc test for comparing multiple groups were applied. Nonparametric data were analyzed using the Mann-Whitney test when comparing two groups, and the Kruskal-Wallis test with Dunn's post hoc test for multiple comparisons. To determine how two factors (genotype and treatment) affect a response variable, we used two-way ANOVA with Tukey's post hoc test. All statistical specifications for each experiment can be found in the figure captions. All data are expressed as the mean ± SEM, and values of $p < 0.05$ were considered statistically significant. Grubbs and ROUT tests were performed to determine the significant outlier values. All graphs and statistical analysis were performed using GraphPad Prism (Version 9.0.0, GraphPad Software, San Diego, California USA). In the *in vivo* studies the sample size was determined based on previous studies in which similar behavioral tests and molecular analysis were performed resulting in significant results (Cabezas-Llobet et al., 2018; García-Forn et al., 2018; Solés-Tarrés et al., 2022).

CHAPTER IV: RESULTS

1. Involvement of PAC1R in PACAP-induced neuroprotection

1.1. PAC1R protein levels are found reduced in STHdhQ111/Q111 cells.

To study the effect of PACAP against mutant huntingtin (mHTT)-mediated toxicity, we used the STHdhQ7/Q7 (Q7) and STHdhQ111/Q111 (Q111) striatal cell lines expressing wild-type (WT) HTT with 7 CAG repeats and mHTT with 111 CAG repeats, respectively. We first characterized the protein levels of PACAP receptors using western blot (Figure 16). We found diminished protein levels of PAC1R and VPAC1R in Q111 cells compared to those in Q7 cells (Figure 16, B; PAC1R: Student's t-test: t=6.257, p=0.0002; VPAC1R: Student's t-test: t=2.574, p=0.0329). Regarding VPAC2R, we did not find significant differences between the cell lines (Figure 16, B; Student's t-test: t=1.160, p=0.2793).

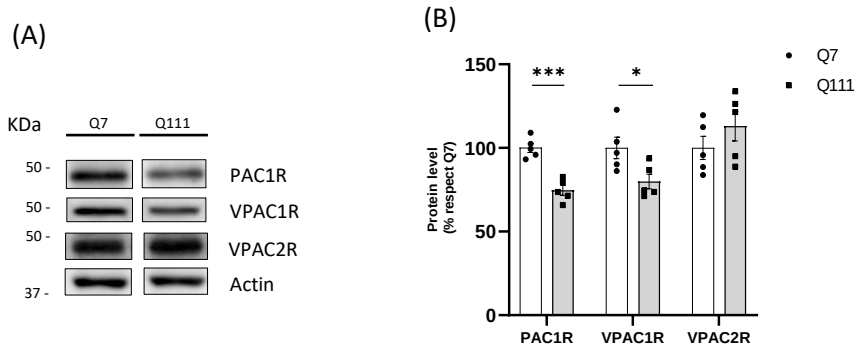


Figure 16. PAC1R expression is reduced in STHdhQ111/Q111 cells. Basal protein levels of PAC1R, VPAC1R and VPAC2R expressed in STHdhQ7/Q7 (Q7) and STHdhQ111/Q111 (Q111) cells were analyzed by western blotting. (**A)** Representative immunoblots are shown. **(B)** Histograms represent the densitometry quantification of protein levels of PAC1R, VPAC1R and VPAC2R normalized to actin for each sample and expressed as a percentage of Q7. Data are presented as the mean ± SEM; n = 5. Data were analyzed using the Student's t-test. *p<0.05, ***p<0.001.

Then, as we previously described that beneficial effects of PACAP are accompanied by the enhancement of PAC1R expression (Cabezas-Llobet et al., 2018), we treated STHdh cells with phosphate-buffered saline (PBS) or PACAP (10^{-7} M) for 24 hours and analyzed PAC1R protein levels (Figure 17). We found a significant interaction between genotype and PACAP treatment (two-way ANOVA interaction effect F (1,16) =10.3, p=0.0055). However, post-hoc test indicated that the addition of PACAP for 24 hours did not induce significant changes in PAC1R protein levels in any of the cellular lines (Figure 17, B). Overall, our results indicate that, as we have described in other HD models (Cabezas-Llobet et al., 2018), PAC1R protein levels are diminished in the presence of mHTT. However, in Q111 cells, PACAP treatment does not increase PAC1R protein levels.

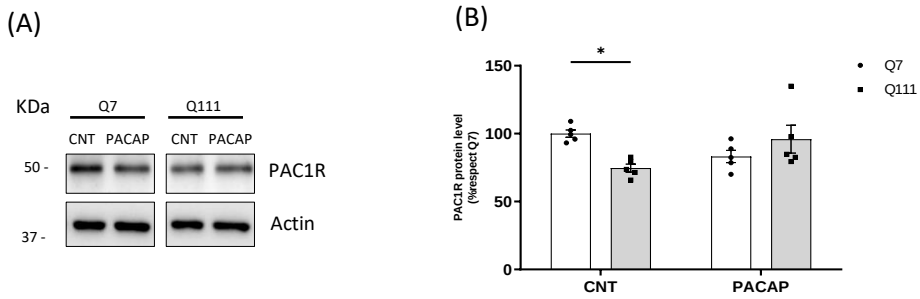


Figure 17. The addition of PACAP does not cause changes in the expression of PAC1R. STHdhQ7/Q7 (Q7) and STHdhQ111/Q111 (Q111) cells were treated with PBS (CNT) or PACAP (10^{-7} M) for 24 hours and protein levels of PAC1R were analyzed using western blotting. **(A)** Representative immunoblots are shown. **(B)** Histograms represent the densitometry quantification of protein levels of PAC1R normalized to actin for each sample and expressed as a percentage of Q7. Data are presented as the mean ± SEM; n = 5. Data were analyzed using two-way ANOVA followed by Tukey as post-hoc test. *p<0.05.

1.2. The activation of PACAP receptors protects from mHTT- mediated cell death.

We then studied the capacity of PACAP to mediate neuroprotection in STHdh cellular model. We treated Q7 and Q111 striatal cell lines with PBS or PACAP (10^{-7} M) for 24 hours in serum-free medium, and then determined the cleaved caspase-3 protein

levels as a marker of apoptosis (Figure 18). We observed that Q111 cells showed significantly higher cleaved caspase-3 levels than Q7 cells (Figure 18, B; two-way ANOVA genotype effect F (1,11) =29.6, p=0.0002), and that the addition of PACAP caused a significant reduction in Q111 cells (Figure 18, B; two-way ANOVA PACAP effect F (1,11) = 36.1, p<0.0001). To specifically explore the role of PAC1R in this action, we compared the capacity of PACAP and VIP to reduce the levels of cleaved caspase-3 (Figure 18). VIP is a peptide with high affinity for VPAC1R and VPAC2R and low affinity for PAC1R. Thus, all effects promoted by PACAP but not by VIP could be attributed to the PAC1R activation. Our results demonstrated that, similar to PACAP, the addition of VIP promoted a significant decrease in cleaved caspase-3 protein levels in Q111 cells (Figure 18, B; two-way ANOVA VIP effect F (1,11) =63.9, p=0<0.0001). We did not found variations in total levels of caspase-3 (Figure 18, A; two-way ANOVA PACAP effect F (1,12) = 2.61, p=0.1321, two-way ANOVA VIP effect F (1,11) = 2.79, p=0.1321). Taken together, these results suggest that all PACAP receptors can initiate an anti-apoptotic cascade.

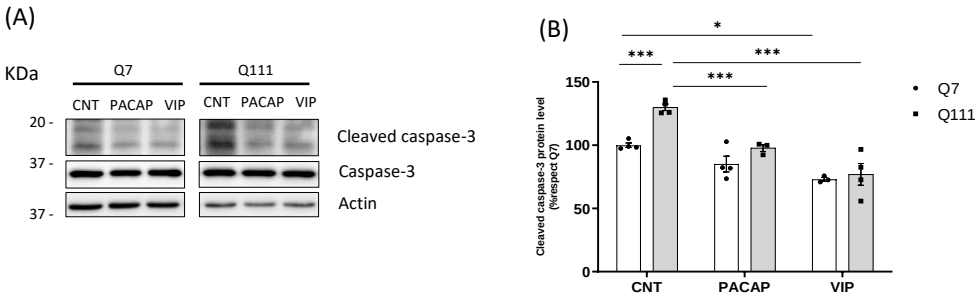


Figure 18. Treatment with PACAP and VIP reduces cleaved caspase-3 levels in STHdhQ111/Q111 cells. STHdhQ7/Q7 (Q7) and STHdhQ111/Q111 (Q111) cells were treated with PBS (CNT), PACAP (10^{-7} M) or VIP (10^{-7} M) for 24 hours in a serum-free medium. Next, cleaved caspase-3 protein levels were measured using western blotting. **(A)** Representative immunoblots are shown. **(B)** Histograms represent the densitometry quantification of cleaved caspase-3 normalized to actin for each sample and expressed as a percentage of Q7-CNT. Data are presented as mean ± SEM; n = 4. Data were analyzed using two-way ANOVA followed by Tukey as a post-hoc test. *p < 0.05 and ***p < 0.001.

1.3. PACAP but not VIP treatment activates the pro-survival pathways ERK1/2 and Akt in STHdhQ111/Q111 cells.

To further explore the PAC1R role in PACAP-mediated neuroprotection, we analyzed the capacity of PACAP and VIP to induce the activation of extracellular signal-regulated kinases 1/2 (ERK 1/2) and Akt (Figure 19), two pro-survival pathways altered in HD (Colin et al., 2005; Bodai and Marsh, 2012). As it had been previously described (Ginés et al., 2010), we observed reduced pERK1/2 and increased pAKt protein levels in Q111 cells compared to Q7 cells (Figure 19, B, C, E, and F; pERK1/2: Student's t-test= 9.410, $p<0.0001$; Figure 19, E-F; pAkt: Welch's t-test: $t=11.90$, $p<0.0001$). Interestingly, in Q111 cells, PACAP treatment significantly affected the protein levels of pERK1/2 (Figure 19, B; one-way ANOVA, $F(5,18) = 2.876$, $p=0.0443$) and pAkt (Figure 19, E; one-way ANOVA, $F(5,18) = 4.275$, $p=0.0097$). Post-hoc Dunnett's test showed that PACAP induced a rapid and transient increase in the protein levels of pERK and pAkt after 15 and 30 minutes of treatment in Q111 cells (Figure 19, B, and D). In Q7 cells, PACAP also increased protein levels of pERK1/2 (Figure 19, B; one-way ANOVA $F(5,18) = 3.770$, $p=0.0164$) after 15 minutes of treatment according to Dunnet's post-hoc test, without any effect on pAkt expression (Figure 19, E; Welch's ANOVA $W(5,6.438) =2.358$, $p=0.1555$). Importantly, after the addition of VIP to Q111 cells, we did not observe significant changes in the protein levels of pERK1/2 (Figure 19, C; one-way ANOVA, $F(5,17) = 2.108$, $p=0.1143$) or pAkt (Figure 19, F; one-way ANOVA $F(5,18) = 0.3876$, $p=0.8508$). In Q7 cells, VIP treatment diminished pERK protein levels after 24 hours of treatment (Figure 19, C; two-way ANOVA interaction effect $F(1,12) =7.20$, $p=0.019$), without any other significant effects (Figure 19, C and F). In conclusion, these results show that PACAP activates the pro-survival pathways ERK1/2 and Akt in Q111 cells by activating PAC1R.

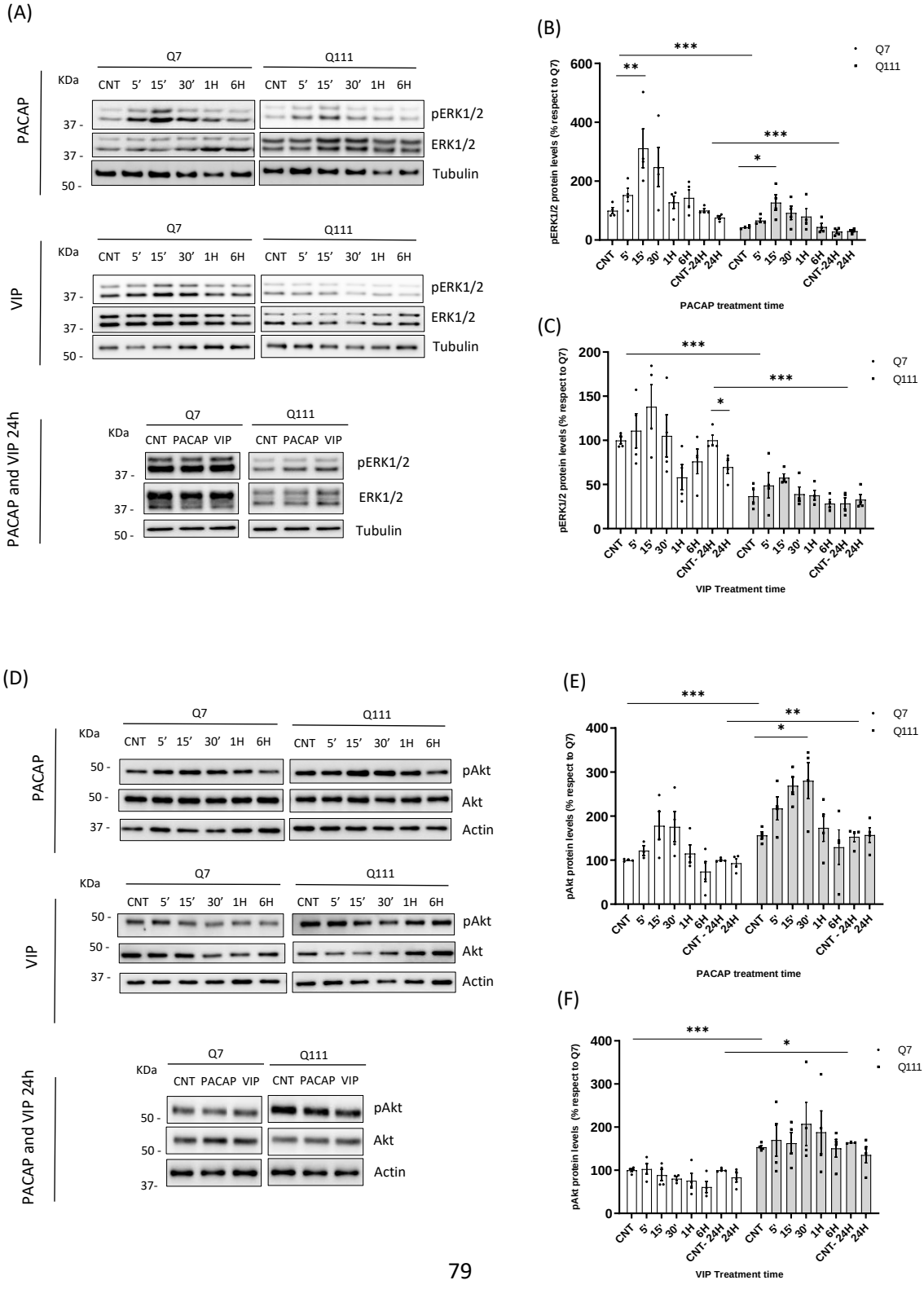

(A)
PACAP
VIP
PACAP and VIP 24h
Q7
Q111
KDa
CNT 5' 15' 30' 1H 6H
CNT PACAP VIP
pERK1/2
ERK1/2
Tubulin
37 -
50 -
(B)
pERK1/2 protein levels (% respect to Q7)
PACAP treatment time
(C)
VIP Treatment time
(D)
pAkt
Akt
Actin
(E)
pAkt protein levels (% respect to Q7)
(F)
pAkt protein levels (% respect to Q7)
CNT 5' 15' 30' 1H 6H CNT-24H 24H
CNT- 24H

Figure 19. PACAP but not VIP treatment promotes the phosphorylation of ERK1/2 and Akt. STHdhQ7/Q7 (Q7) and STHdhQ111/Q111 (Q111) cells were treated with PBS (CNT), PACAP (10-7M) or VIP (10-7M) for 5, 15, 30 minutes, 1, 6 and 24 hours. Protein levels of pERK1/2 and pAkt (Ser473) were analyzed by western blotting. **(A** and **D)** Representative immunoblots are shown. Histograms represent the densitometry quantification of **(B-C)** pERK1/2 and **(E-F)** pAkt normalized to actin or tubulin for each sample and expressed as a percentage of Q7-CNT. Data are represented as the mean ± SEM; n = 4. Differences in protein basal levels between Q7-CNT and Q111-CNT cells were analyzed by unpaired Student's t-test. The effect of PACAP and VIP treatments for 24 hours was analyzed by using the two-way ANOVA test followed by Tukey as post-hoc test. Treatment response over time was analyzed separately in each cell type using one-way ANOVA followed by Dunnett as post-hoc test. When variances between groups were not equal Welch's correction was applied and Tamhane's T2 test was performed *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

1.4. Activation of ERK1/2 and Akt mediates, at least in part, the anti-apoptotic effect of PACAP.

To determine whether the activation of pERK1/2 and pAkt initiated by PACAP in Q111 cells is involved in the anti-apoptotic effect observed, we treated Q7 and Q111 cells with pharmacological inhibitors of ERK and Akt, PD98059 (PD) and LY294002 (LY), respectively, for 30 minutes before treatment with PACAP. After 24 hours of PACAP addition, we analyzed cleaved caspase-3 levels (Figure 20). As we previously observed, we found that treatment with PACAP induces a significant reduction of cleaved caspase-3 protein levels in Q111 cells (Figure 20, B; PD: Welch's ANOVA W (3,5.705) =10.88, p=0.088; LY: Welch's ANOVA W (3,5.379) =8.958, p=0.0159) without significant changes in Q7 cells (Figure 20, B; PD: Welch's ANOVA W (3,6.066) =0.8809, p=0.5016; LY: Welch's ANOVA W (3,6.444) =0.1932, p=0.8975). In Q111 cells treated with inhibitors and PACAP, the protein levels of cleaved caspase-3 tended to decrease. However, post-hoc Tamhane's T2 test showed significant group differences only between Q111-VEH and Q111-PACAP, indicating that treatment with PD and LY blocked the effects of PACAP on cleaved caspase-3 levels (Figure 20, B). Overall, we can conclude that ERK1/2 and Akt, two signaling pathways linked to PAC1R, are

involved in the PACAP-mediated inhibition of caspase-3, but it is likely that other pathways play a role in anti-apoptotic activity.

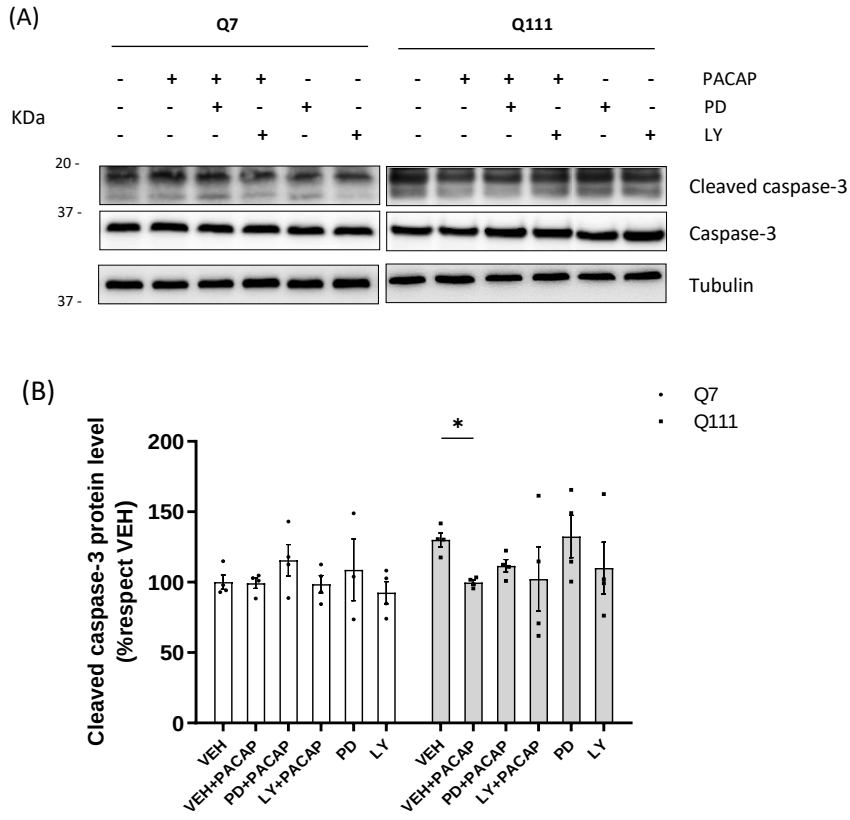


Figure 20. ERK1/2 and Akt pathways mediate the PACAP inhibitory effect on cleaved caspase-3 protein levels. STHdhQ7/Q7 (Q7) and STHdhQ111/Q111 (Q111) cells were treated with DMSO (VEH), PD98059 (PD, 10 µM) or LY94002 (LY, 10 µM). PACAP treatment was performed 30 minutes after and 24 hours later, protein levels of cleaved caspase-3 were analyzed **(A)** Representative immunoblots are shown. Histograms represent the densitometry quantification of **(B)** cleaved caspase-3 normalized to tubulin for each sample and expressed as a percentage of Q7-VEH. Data are represented as the mean ± SEM; n = 4. Treatment response was analyzed separately in each cell type and for each inhibitor. Data were analyzed by using one-way ANOVA followed by Tukey as post-hoc test. When variances between groups were not equal Welch's correction was applied and Thamane's T2 was used as a post hoc test. *$p < 0.05$.

1.5. PACAP enhances the expression of neurotrophic proteins in STHdhQ111/Q111 cells by the activation of PAC1R.

Finally, we studied the involvement of PAC1R in the PACAP-mediated expression of proteins related to neuronal viability, synaptic function, and neurotrophism. Thus, we treated Q7 and Q111 striatal cell lines with PBS, PACAP (10^{-7} M), or VIP (10^{-7} M) for 24 hours and studied the protein levels of c-fos, early growth response 1 (egr1), cAMP-response element- binding protein (CREB) -binding protein (CBP), and brain-derived neurotrophic factor (BDNF) using western blot (Figure 21). We chose these proteins because we previously showed that PACAP can rescue its levels in the hippocampus and striatum of the R6/1 mouse model (Cabezas-Llobet et al., 2018; Solés-Tarrés et al., 2022). We did not find no significant differences in the expression of any of these proteins between Q7 and Q111 cells (Figure 21, B-E; c-fos: two-way ANOVA genotype effect $F(1,16) = 0.2278$, $p=0.6396$; egr-1: two-way ANOVA genotype effect $F(1,15) = 0.00556$, $p=0.9415$; CBP: two-way ANOVA genotype effect $F(1,16) = 1.75$, $p=0.2046$; BDNF: two-way ANOVA genotype effect $F(1,16) = 3.888$, $p=0.0662$). However, PACAP treatment had a significant effect in protein levels of c-fos (Figure 21, B; two-way ANOVA PACAP effect $F(1,16) = 8.778$, $p=0.0092$), egr1 (Figure 21, C; two-way ANOVA PACAP effect $F(1,15) = 5.55$, $p=0.0325$), and CBP (Figure 21, D; two-way ANOVA PACAP effect $F(1,15) = 5.55$, $p=0.0325$). We also found a significant interaction between genotype and PACAP treatment on BDNF protein levels (Figure 21, E; two-way ANOVA interaction effect, $F(1,16) = 12.19$, $p=0.0030$). Interestingly, Tukey's post-hoc test demonstrated that PACAP significantly increased the protein levels of all these proteins in Q111 cells without affecting Q7 cells (Figure 21, B-E). In contrast, we found that VIP treatment did not affect levels of these proteins in Q111 cells (Figure 21, B- E; c-fos: two-way ANOVA VIP effect $F(1,16) = 1.846$, $p=0.1931$; egr1: two-way ANOVA VIP effect $F(1,16) = 1.19$, $p=0.2905$; CBP: two-way ANOVA VIP effect $F(1,16) = 1.03$, $p=0.3246$; BDNF: two-way ANOVA VIP effect $F(1,15) = 4.497$, $p=0.0510$). Altogether,

we can conclude that the PACAP-mediated increase of c-fos, egr1, CBP, and BDNF protein levels in Q111 cells is due to PAC1R activation.

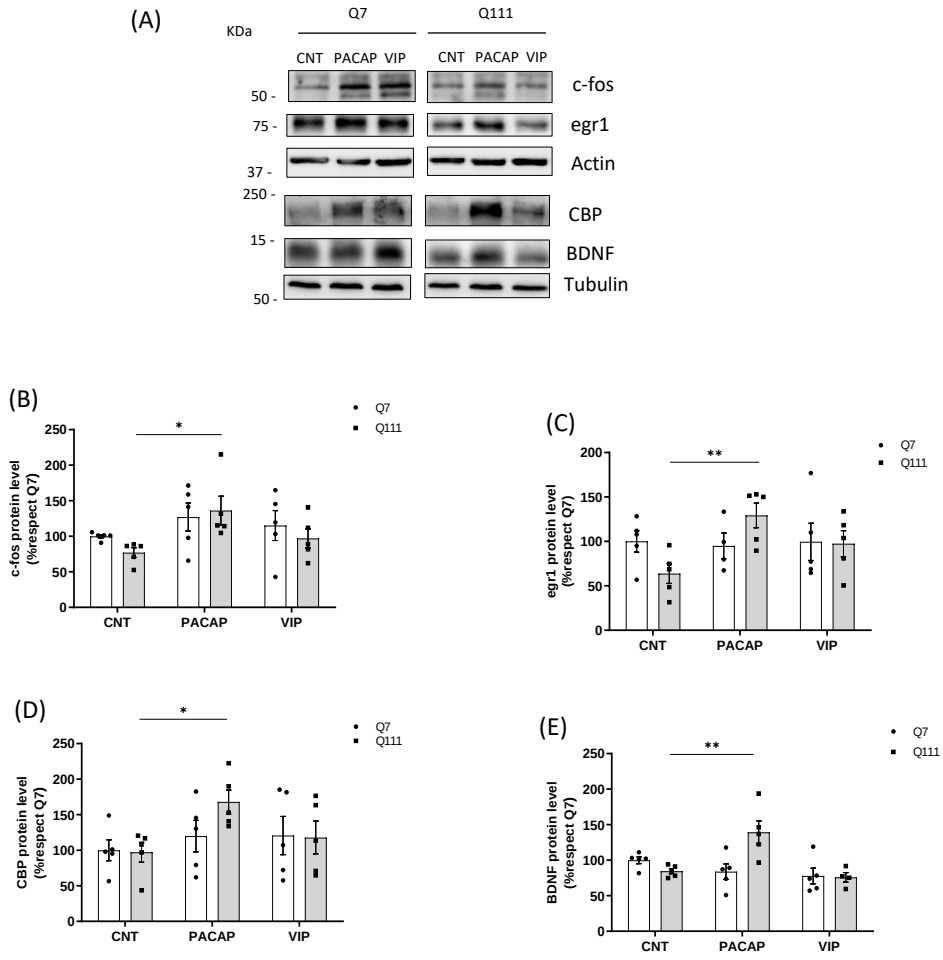


Figure 21. PACAP but not VIP treatment increases c-fos, egr1, CBP, and BDNF levels in STHdhQ111/Q111 cells. STHdhQ7/Q7 (Q7) and STHdhQ111/Q111 (Q111) cells were treated with PBS (CNT), PACAP (10^{-7} M) or VIP (10^{-7} M). Protein levels of c-fos, egr1, and CBP were analyzed 6 hours after treatment and BDNF protein levels 24 hours after treatment. **(A)** Representative immunoblots are shown. Histograms represent the densitometry quantification of **(B)** c-fos, **(C)** egr1, **(D)** CBP, and **(E)** BDNF normalized to actin or tubulin for each sample and expressed as a percentage of Q7-CNT. Data are represented as the mean ± SEM; n = 5. Data was analyzed by using two-way ANOVA followed by Tukey as post-hoc test. *p < 0.05, **p < 0.01.

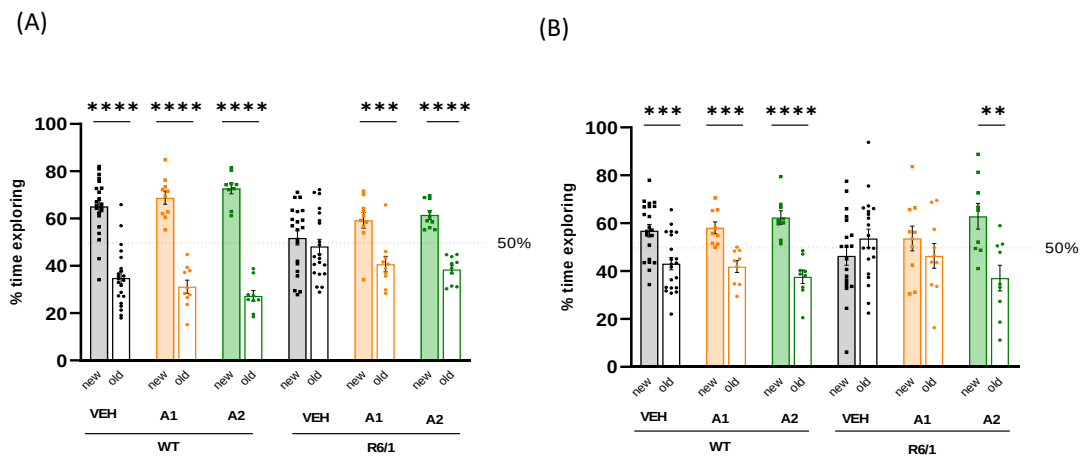
2. Therapeutic potential of PACAP analogues in hippocampal neuropathology and associated cognitive disturbances.

2.1. Intranasal administration of PACAP analogues improves spatial memory deficits in R6/1 mice.

Since we have previously described that PACAP enhances hippocampal synaptic plasticity and memory in HD (Cabezas-Llobet et al., 2018) we investigated whether A1 and A2 also have the capacity to fight memory deficits in R6/1 mice. At 13 weeks of age, we performed a daily intranasal administration of PBS, A1, or A2 (30 µg/kg/ day) to WT and R6/1 mice for twelve days. From the 8th day of treatment, we subjected the animals to T-MAZE test and novel object location test (NOLT), both designed to assess hippocampal-dependent spatial memory. In the T-MAZE test with an inter-trial interval (ITI) of 1 hour (Figure 22, A), we found that while WT mice spent more time exploring the new arm (t=9.339, p<0.0001), this preference was lost in R6/1, which explored both arms equally (t=0.8488, p=0.4010). Importantly, when R6/1 mice were treated with A1 or A2, they showed an exploratory preference, spending more time in the new arm (R6/1 A1: t=3.985, p=0.0009; R6/1 A2: t=8.989, p<0.0001). As expected, WT animals treated with A1 or A2 preserved their preference for the new arm (WT A1, t=9.634, p<0.0001; WT A2, t=14.07, p<0.0001). We then used the NOLT test with an ITI of 24 hours to explore the capacity of PACAP analogues to preserve spatial memory for longer periods of time (Figure 22, B). We found that WT mice, treated or untreated with peptides, spent more time exploring the object situated in the new location than the one in the old location (WT VEH: t=3.811, p=0.0005; WT A1: t=4.680, p=0.0003; WT A2: t=6.247, p<0.0001). R6/1 mice did not show this preference, exploring the two objects equally (t=1.331, p=0.1911). Remarkably, R6/1 mice treated with A2, but not with A1, spent significantly more time exploring the new arm (R6/1 A1: t=0.9955, p=0.3327; R6/1 A2: t=3.381, p=0.0038). In conclusion, these results revealed that

spatial memory deficits in R6/1 mice can be rescued by PACAP analogues, with being A2 effective in improving memory for longer periods of time.

To analyze whether general locomotor activity and anxiety behavior were affected by treatment with PACAP analogues, animals were also subjected to the open field test (Figure 22, C-D). We measured the total distance travelled in 30 minutes to evaluate locomotor activity and the time spent in the central area as a measure of anxiety levels. We found that R6/1 mice displayed reduced locomotor activity (Figure 22, C; two-way ANOVA genotype effect for total travelled distance F (1,69) = 16.7, p=0.0001) and increased time in center (Figure 22, D; two-way ANOVA genotype effect for percentage of time in center F (1,71) =5.11, p=0.0268) compared with WT mice. Importantly, intranasal administration of A1 and A2 did not induce significant changes in these parameters (Figure 22, C-D; two-way ANOVA treatment effect for total travelled distance F (2,69) = 1.66, p=0.1976; two-way ANOVA treatment effect for percentage of time in center F (1,71) =0.0310, p=0.9695), indicating that PACAP analogues did not have any significant effect on locomotion and anxiety behavior.



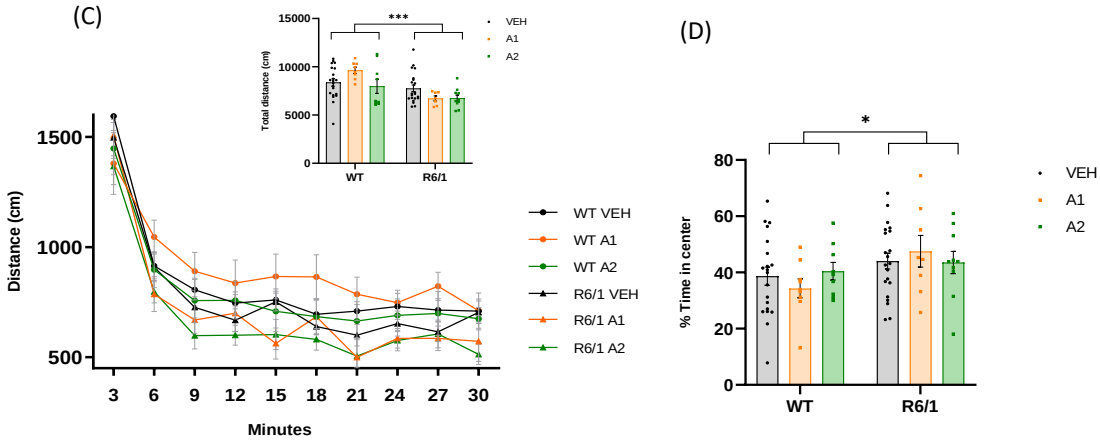


Figure 22. Treatment with A1 and A2 prevents spatial-memory deficits in R6/1 mice. WT and R6/1 mice were treated daily for twelve days with PBS as vehicle (VEH), A1, and A2 at 13 weeks of age. A battery of behavioral tests was performed during the second week of treatment. **(A)** In T-MAZE test, we calculated the percentage of time exploring old and new arm 1 hour after the training session **(B)** In the NOLT test, we calculated the percentage of time exploring objects in the old and new location 24 hours after the training session. **(C)** To assess locomotor activity, total distance travelled, and total distance travelled each 3 minutes were measured **(D)** To evaluate stress behavior, the percentage of time spent in the center was calculated. Data are represented as the mean ± SEM. Number of animals per group: WT VEH: 21, WT A1: 10, WT A2: 9, R6/1, VEH: 21, R6/1 A1: 10, R6/1 A2:10. In A and B, data were analyzed separately for each group performing the Student's t-test. In C and D data were analyzed by using two-way ANOVA followed by Tukey as post-hoc test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

2.2. Dendritic spine density is increased in CA1 hippocampal region of R6/1 mice treated with PACAP analogues.

To assess whether structural synaptic plasticity underlies behavioral improvements in R6/1 mice treated with A1 or A2, we analyzed spine density in CA1 dendrites using the Golgi-Cox method (Figure 23). Our results showed diminished dendritic spine density in CA1 pyramidal neurons of R6/1 mice (Figure 23, C; two-way ANOVA genotype effect, $F(1,565) = 182$, $p<0.0001$). Importantly, treatments with A1 and A2 had a significant effect on the number of dendritic spines (Figure 23, C; two-way ANOVA treatment effect, $F(2,565) = 21.9$, $p<0.0001$). Tukey's post hoc analysis showed that both analogues increased dendritic spine density in R6/1 mice but had no effect in WT mice

(Figure 23, C). Taken together, we can affirm that treatments with A1 and A2 can rescue dendritic spine loss in the hippocampal CA1 region of the R6/1 mice.

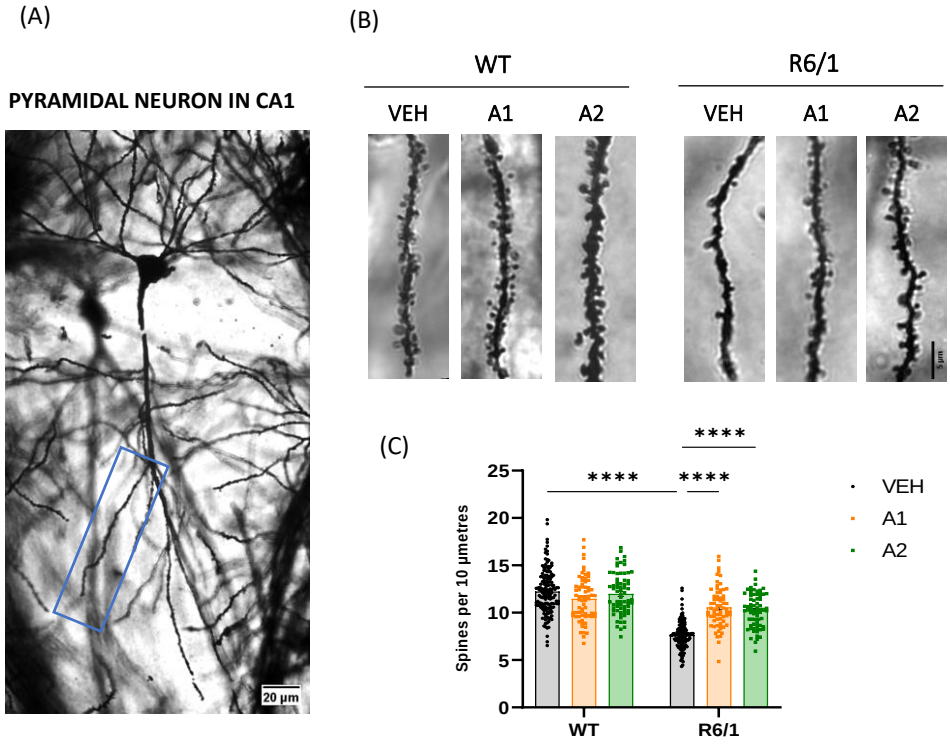
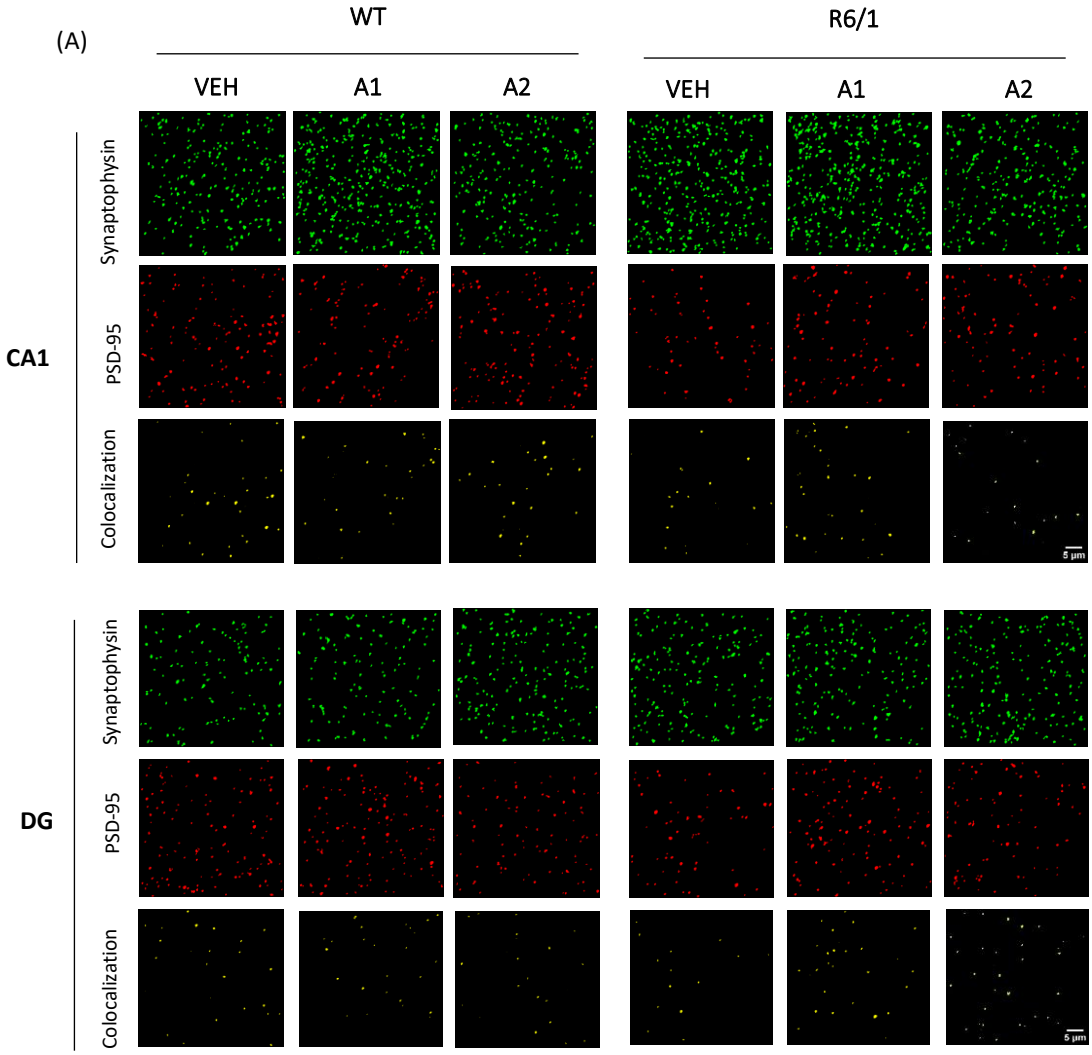


Figure 23. Treatment with A1 and A2 prevents dendritic spine loss in R6/1 mice. Dendritic spine density in the hippocampal CA1 of WT and R6/1 mice, treated with PBS as vehicle-(VEH), A1 or A2, was determined using the Golgi staining **(A)** Illustrative image of a Golgi-impregnated pyramidal neuron from the CA1 region of WT VEH mice hippocampus. **(B)** Representative segments of dendrites from VEH-, A1-, and A2- treated WT and R6/1 mice. **(C)** Quantitative analysis of spine densities per 10 µm of dendritic length in 5–10 neurons per mouse. Data are presented as the mean ± SEM. Number of samples: WT VEH: 155 dendrites from 12 mice, WT A1: 68 dendrites from 5 mice, WT A2: 70 dendrites from 6 mice, R6/1 VEH: 143 dendrites from 11 mice, R6/1 A1:67 dendrites from 5 mice, R6/1 A2: 70 dendrites from 6 mice. Data were analyzed using two-way ANOVA followed by Tukey's post-hoc test. ****p<0.0001.

2.3. Intranasal administration of PACAP analogues increases the number of excitatory synapses in the hippocampus of R6/1 treated mice.

Considering the results obtained in spine density, we analyze the effects of A1 and A2 treatments in the number of excitatory synapses in hippocampal CA1, CA3, and DG regions. Antibodies against synaptophysin and postsynaptic density protein 95 (PSD-95) were used to visualize and count the number of presynaptic (green) and postsynaptic (red) particles, respectively. We also counted the colocalization of synaptophysin and PSD-95, representing the number of excitatory synapses (yellow) (Figure 24). Our results indicated no significant differences in the number of synaptophysin-positive particles between WT and R6/1 mice in any region of the hippocampus (Figure 24, B-D; CA1: two-way ANOVA genotype effect $F(1,38) = 2.207$, $p=0.146$; CA3: two-way ANOVA genotype effect $F(1,38) = 0.2395$, $p=0.627$; DG: two-way ANOVA genotype effect $F(1,38) = 0.7926$, $p=0.780$). In contrast, R6/1 mice showed a significant reduction in the number of PSD-95 positive particles (Figure 24, E-G; CA1: two-way ANOVA genotype effect $F(1,38) = 42.13$, $p<0.001$; CA3: two-way ANOVA genotype effect $F(1,37) = 15.40$, $p<0.001$; DG: two-way ANOVA genotype effect $F(1,38) = 10.79$, $p=0.002$). Interestingly, we found a significant reduction in the number of excitatory synapses in R6/1 mice in all hippocampal regions analyzed (Figure 24, H-J; CA1: two-way ANOVA genotype effect $F(2,37) = 17.96$, $p<0.001$; CA3 two-way ANOVA genotype effect $F(1,37) = 6.656$, $p=0.014$; DG: two-way ANOVA genotype effect $F(1,38) = 11.30$, $p=0.02$). Importantly, treatment with PACAP analogues did not induce significant changes in the number of synaptophysin and PSD-95 particles in most of the analyzed regions. Only the number of synaptophysin particles in CA1 was significantly affected by treatment (Figure 24, B; two-way ANOVA treatment effect, $F(2,38) = 5.011$, $p=0.012$). However, the analysis of synaptophysin and PSD-95 colocalization indicated that treatment with analogues had a significant effect on the number of synapses in all hippocampal regions analyzed (Figure 24, H-J; CA1: two-way ANOVA treatment effect $F(2,38) = 5.011$, $p=0.012$; CA3 two-way ANOVA treatment

effect F (2,37) = 4.748, p=0.015; DG: two-way ANOVA treatment effect F (2,38) = 6.206, p=0.005). Importantly, Tukey post-hoc test demonstrated that A1 increased the number of excitatory synapses in R6/1 hippocampal CA1 (Figure 24, H), while A2 did it in DG (Figure 24, J). The post-hoc analysis also showed that there were no significant differences in any of the parameters studied in WT-treated mice with respect to WT VEH (Figure 24, B-J). In conclusion, treatment with A1 and A2 increased synaptic density in different regions of the hippocampus, with a principal effect of A1 in CA1 and a main effect of A2 in DG.



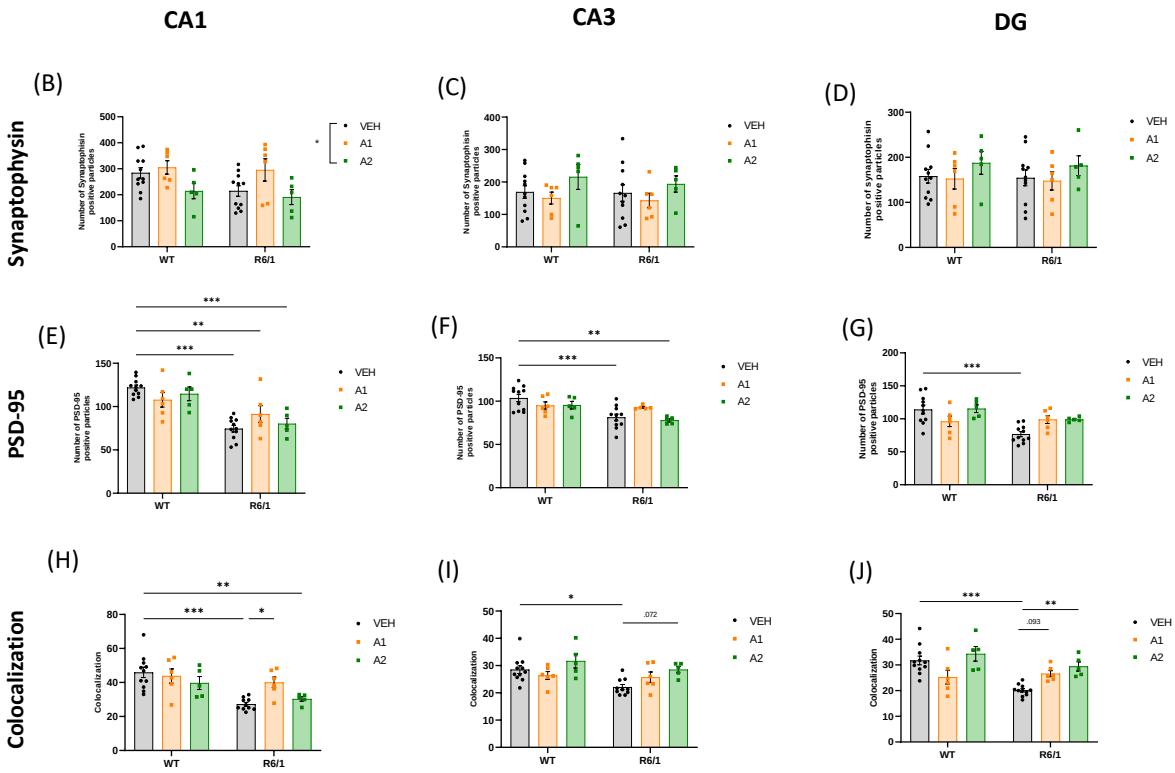


Figure 24. Treatments with A1 and A2 increase the number of synaptic particles in different regions of the hippocampus in R6/1 mice. Brain samples from WT and R6/1 mice treated with PBS as vehicle (VEH), A1 or A2, were used for immunofluorescence staining against synaptophysin and PSD-95. At least three 40-µm slices containing dorsal hippocampus were analyzed per mouse. Two representative images of the CA1 and CA3- *stratum radiatum* and of the DG- *stratum moleculare* were obtained from each slice. The number of individual synaptic puncta (pre- and post-synaptic) was counted per field using the optimized threshold protocols selected within the ImageJ. Then, the software identified the co-localization of synaptic puncta as the overlap of 1 pixel or more. **(A)** Representative masks of images obtained by labelling synaptophysin (green) and PSD-95 (red), and its colocalization (yellow) in the *stratum radiatum* of hippocampal CA1 and in molecular layer of DG. Histograms represent the quantification of the **(B-D)** number of synaptophysin-positive particles, **(E-G)** number of PSD-95 positive particles and **(H-J)** number of synaptophysin and PSD-95 colocalization from *stratum radiatum* of hippocampal CA1 and CA3, and in molecular layer of DG respectively. Data are presented as the mean ± SEM. Number of animals per group: WT VEH: 11, WT A1: 6, WT A2: 5, R6/1, VEH: 11, R6/1 A1: 6, R6/1 A2:5. All data were analyzed using two-way ANOVA followed by Tukey's post-hoc test. *p<0.05, **p<0.01, ***p<0.001.

2.4. Intranasal administration of A1 modulates hippocampal NMDAR function and localization in R6/1 mice.

Because of its implication in hippocampal long-term potentiation (LTP), we studied whether treatment with analogues induces changes in NMDAR phosphorylation and total levels (Figure 25). We restricted our study to GluN2A and GluN2B subunits since they are predominant in mature synapses and their involvement in hippocampus-dependent spatial memory has been demonstrated (Cull-Candy et al., 2001; Cull-Candy and Leszkiewicz, 2004; Zhang et al., 2013). We first analyzed the phosphorylation of GluN2A at tyrosine 1246 (Y1246) and tyrosine 1325 (Y1325), which enhance the current flux through NMDAR (Köhr and Seeburg, 1996; Taniguchi et al., 2009). We found significant differences in both phosphorylated GluN2A subunits between the genotypes (Figure 25, B-C; p(Y1246) GluN2A: two-way ANOVA genotype effect $F(1,53) = 17.2$, $p=0.0001$; (Y1325) GluN2A: two-way ANOVA genotype effect $F(1,52) = 12.8$, $p=0.0008$). Importantly, Tukey's post-hoc test showed a significant increase in both phosphorylated forms of GluN2A in R6/1 mice treated with A1 compared to WT mice treated with vehicle (Figure 25, B-C). Related to GluN2A total levels, we found significant differences between genotypes (Figure 25, D; two-way ANOVA genotype effect, $F(1,54) = 5.72$, $p=0.0202$). In addition, treatment with analogues induced significant changes in the total levels of GluN2A (Figure 25, D; two-way ANOVA treatment effect, $F(2,54) = 6.02$, $p=0.0044$) although the post-hoc test did not show significant differences between groups of interest. Regarding the GluN2B subunit, it is important to mention that phosphorylation of tyrosine 1472 (Y1472) allows GluN2B containing NMDAR to be placed at the postsynaptic density, facilitating the LTP (Rostas et al., 1996; Prybylowski et al., 2005; Hallett et al., 2006). We did not find significant differences between genotypes in phosphorylated levels of GluN2B (Figure 25, E; two-way ANOVA genotype effect $F(1,54) = 0.136$, $p=0.7133$). Importantly, treatment with the analogues induced significant changes in the phosphorylated levels of GluN2B (Figure 25, E; two-way ANOVA treatment effect $F(2,54) = 11.6$, $p<0.001$) and Tukey's

post hoc test showed that A1 induced a significant increase in p(Y1472) GluN2B protein levels in WT and R6/1 mice (Figure 25, E). We also found genotype differences in total levels of GluN2B (Figure 25, F; two-way ANOVA genotype effect F (1,53) = 6.60, p=0.0131). Moreover, we observed a significant increase in GluN2B total protein levels in R6/1 mice treated with A1 compared with WT mice treated with vehicle (Figure 25, F). In conclusion, treatment with A1 induced changes in the phosphorylation and total levels of the GluN2A and GluN2B subunits of NMDARs.

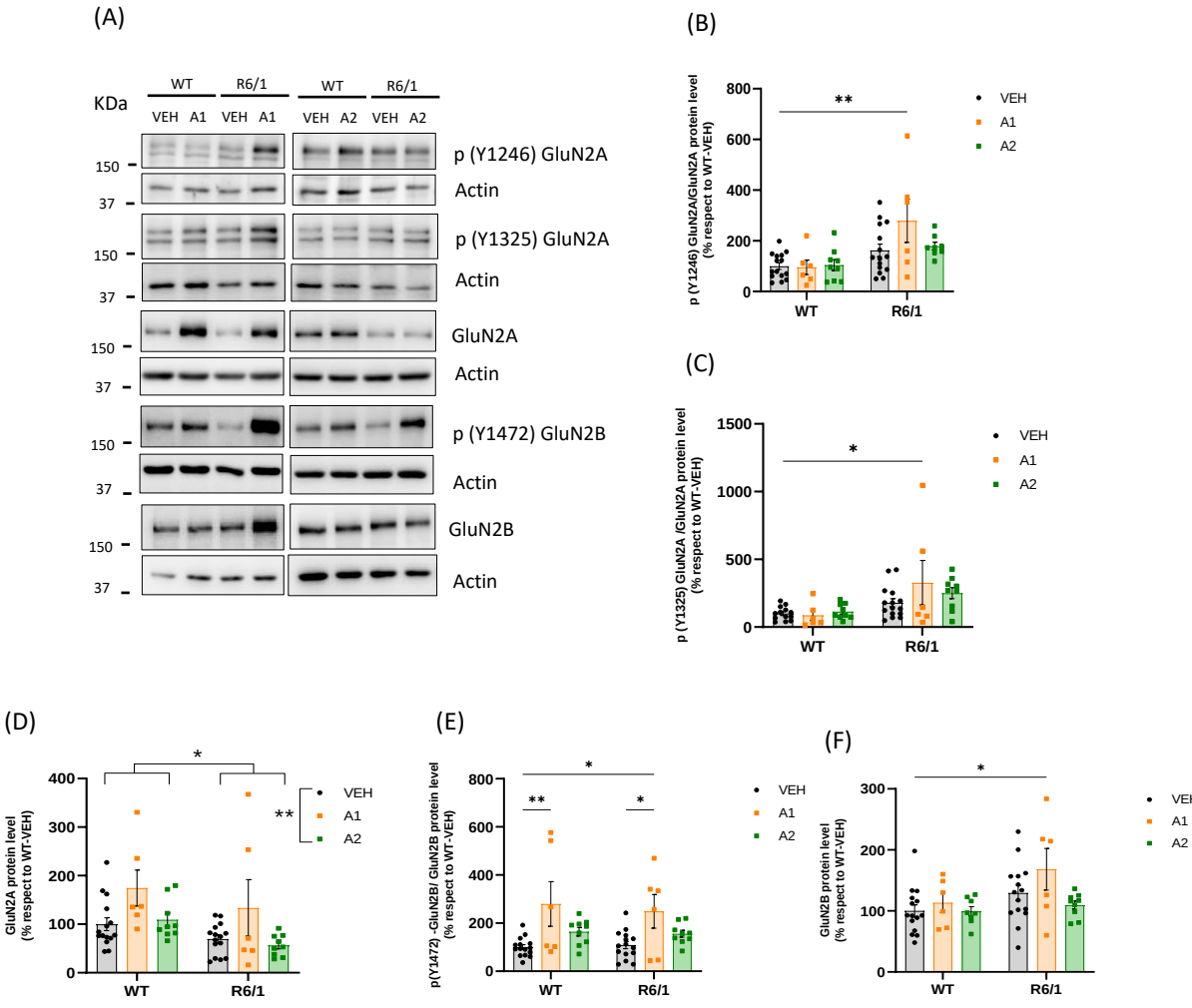


Figure 25. Treatment with A1 induces changes on phosphorylation and total levels of GluN2B and GluN2A subunits of NMDAR. Brain samples from WT and R6/1 mice treated with PBS as vehicle (VEH), A1 or A2, were used for studying hippocampal protein levels p(Y1246) GluN2A, p(Y1325) GluN2A, GluN2A, p(Y1472) GluN2B, and GluN2B **(A)** Representative images of immunoblotting are shown. Histograms represent the densitometry quantification of **(B)** p(Y1246) GluN2A, **(C)** p(Y1325) GluN2A, **(D)** GluN2A, **(E)** p(Y1472) GluN2B and **(F)** GluN2B normalized to actin, GluN2B or GluN2A for each sample and expressed as percentage of WT VEH. Data are presented as the mean ± SEM. Number of animals per group: WT VEH: 15, WT A1: 6, WT A2: 9, R6/1, VEH: 15, R6/1 A1: 6, R6/1 A2:9. All data were analyzed using two-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. *p<0.05, **p<0.01.

2.5. Intranasal administration of PACAP analogues does not change hippocampal protein levels of BDNF and its receptors TrkB and p75NTR.

We previously reported that PACAP has a neurotrophic effect that induces BDNF expression in R6/1 mice and STHdh cellular models of HD (Cabezas-Llobet et al., 2018; Solés-Tarrés et al., 2022). Since BDNF can influence the induction of synaptic plasticity through the signaling related to its receptors, we studied whether PACAP analogues promote the expression of BDNF or change the expression of its receptors named tropomyosin receptor kinase B (TrkB) and p75 neurotrophin receptor (P75NTR) in the hippocampus of R6/1 mice (Figure 26). We found a significant decrease in BDNF protein levels without differences in TrkB and p75NTR in R6/1 mice compared to WT mice (Figure 26, B-D; BDNF: two-way ANOVA genotype effect $F(1,46) = 29.4$, $p<0.001$; TrkB: two-way ANOVA genotype effect $F(1,41) = 0.177$ $p=0.6762$, p75NTR: two-way ANOVA genotype effect $F(1,53) = 0.177$, $p=0.6116$). Treatment with A1 or A2 did not induce any significant changes in the expression of BDNF and p75NTR (Figure 26, B and D; BDNF: two-way ANOVA treatment effect $F(2,46) = 2.02$, $p=0.1445$; p75NTR: two-way ANOVA treatment effect $F(2,53) = 1.16$, $p=0.4299$). However, in the case of TrkB, ANOVA showed an interaction between genotype and treatment (Figure 26, C; two-way ANOVA interaction $F(2,41) = 3.82$, $p=0.0300$), and a clear tendency of the reduction of TrkB in WT animals treated with A1 (Figure 26, C).

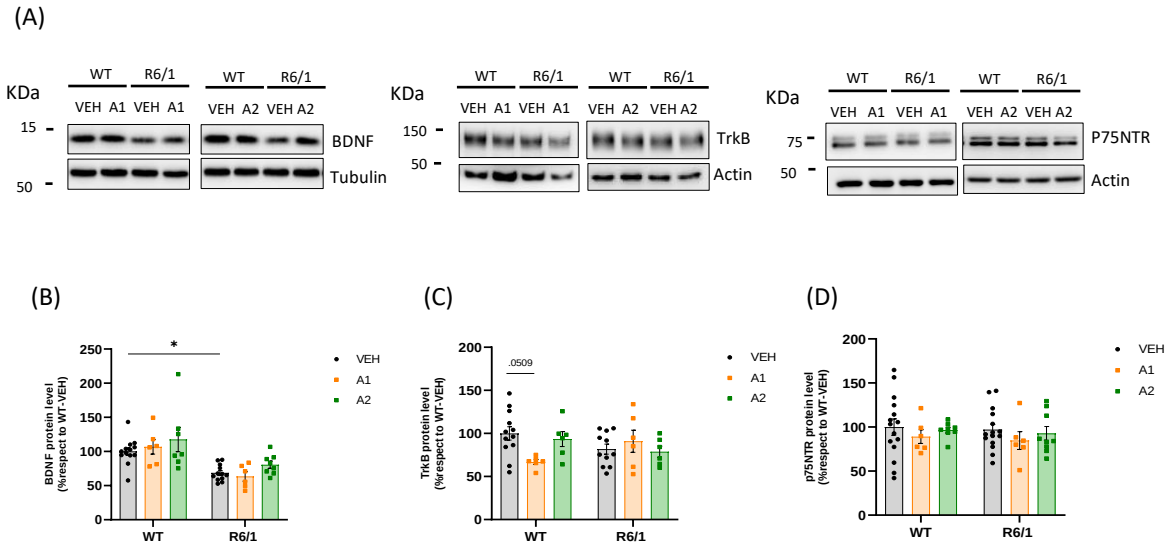


Figure 26. Treatment with PACAP analogues did not change the hippocampal protein levels of BDNF and its receptors TrkB and p75NTR. Brain samples from WT and R6/1 mice treated with PBS as vehicle (VEH), A1, or A2, were used to study hippocampal protein levels of BDNF, TrkB, and P75NTR by western blotting. **(A)** Representative images of immunoblotting are shown. Histograms represent the densitometry quantification of **(B)** BDNF, **(C)** TrkB and **(D)** P75NTR normalized to tubulin or actin for each sample and expressed as a percentage of WT VEH. Data are presented as mean ± SEM. Number of animals per group: WT VEH: 12-15, WT A1: 6, WT A2: 6-8, R6/1, VEH: 11-12, R6/1 A1: 6, R6/1 A2:6-9. All data were analyzed using two-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. *p<0.05.

2.6. Intranasal administration of A2 increases pCREB protein levels in hippocampal DG of R6/1 treated mice.

Signalling pathways associated with PACAP receptors can activate CREB (Baxter et al., 2011; Ye et al., 2019), which is essential for activity-induced gene expression and memory formation (Silva et al., 1998; Barco et al., 2005). Therefore, we analysed by immunofluorescence whether PACAP analogues had the capacity to increase pCREB intensity in the CA1, CA3, and DG hippocampal regions (Figure 27, A-D).

Our results showed that R6/1 mice displayed significantly reduced intensity of pCREB compared with WT mice in the CA3 hippocampal region, with no differences in CA1

and DG regions (Figure 27, B–D; CA1: two-way ANOVA genotype effect $F(1,38) = 2.91$, $p=0.0961$; CA3: two-way ANOVA genotype effect $F(1,35) = 24.2$ $p=0<0.0001$; DG: two-way ANOVA genotype effect $F(1,38) = 0.330$, $p=0.5691$). Interestingly, pCREB intensity was significantly affected by treatment in all the regions analyzed (Figure 27, B-D; CA1: two-way ANOVA treatment effect $F(2,38) = 6.36$, $p=0.004$; CA3: two-way ANOVA treatment effect $F(2,35) = 8.98$, $p=0.0007$; DG: two-way ANOVA treatment effect $F(2,38) = 18.2$, $p<0.0001$). However, Tukey's post-hoc test showed that only treatment with A2 significantly increased pCREB intensity specifically in pyramidal neurons from the CA3 region of WT animals and granule neurons of the DG of R6/1 mice (Figure 27, C and D). We also found that the pCREB intensity tended to increase in the CA3 hippocampal region of R6/1 mice after A2 treatment (Figure 27, C). Then, since we previously found that the beneficial effects of PACAP were associated with an increase in the protein levels of CBP (Cabezas-Llobet et al., 2018; Solés-Tarrés et al., 2022), the coactivator of CREB, we studied if treatment with PACAP analogues has any effect on the expression of this protein (Figure 27, E-F). Our results showed differences in CBP protein levels between the genotypes (Figure 27, F; two-way ANOVA genotype effect, $F(1,53) = 9.61$, $p=0.0031$). However, we did not observe significant changes in the protein levels of CBP after treatment with the PACAP analogues (Figure 27, F; two-way ANOVA treatment effect, $F(2,53) = 0.143$, $p=0.8667$). Taken together, we can conclude that only A2 enhanced CREB activation. This effect was region-specific, as it was only significant in DG of R6/1 and in CA3 of WT. In addition, A2-mediated CREB activation was not associated with increased expression of its coactivator, CBP.

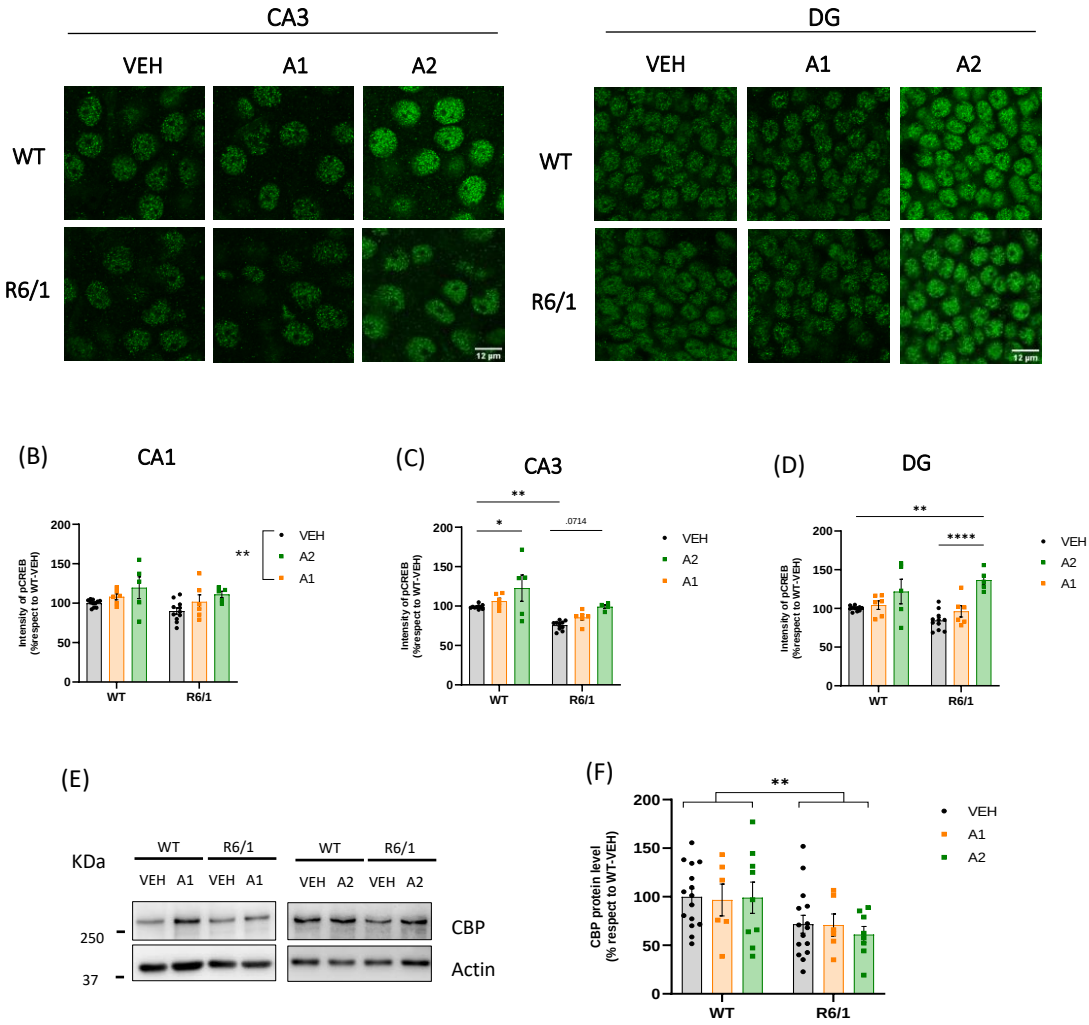


Figure 27. Treatment with A2 enhances pCREB expression in the DG hippocampal region of R6/1 mice without increasing CBP protein levels. Brain samples from WT and R6/1 mice treated with PBS as vehicle (VEH), A1, or A2 were used for immunofluorescence staining of pCREB. **(A)** Representative immunofluorescence images showing pCREB in green in the hippocampal CA3 and DG. Histograms represent the quantification of **(B-D)** pCREB intensity expressed as a percentage of WT VEH in the CA1, CA3, and DG hippocampal regions. Data are presented as mean ± SEM. Number of animals per group: WT VEH: 11, WT A1: 6, WT A2: 5, R6/1, VEH: 10-11, R6/1 A1: 6, R6/1 A2: 4-5. Brain samples from WT and R6/1 mice treated with PBS, A1, or A2 were also used to study the hippocampal protein levels of CBP by western blotting. **(E)** Representative images of immunoblotting are shown. Histograms represent densitometry quantification of **(F)** CBP normalized to actin for each sample and expressed as a percentage of WT VEH. Data are presented as mean ± SEM. Number of animals per group: WT VEH: 15, WT A1: 6, WT A2: 9, R6/1, VEH: 15, R6/1 A1: 6, R6/1 A2: 8. All data were analyzed using two-way analysis of variance (ANOVA) followed by Tukey's

2.7. Beneficial effects of PACAP analogues are not associated with the increase of PAC1R protein levels.

In previous studies, we found that PAC1R protein levels were reduced in the hippocampus of R6/1 mice, and that the beneficial effects of PACAP were associated with an increase in PAC1R (Cabezas-Llobet et al., 2018). Therefore, we studied PAC1R levels after treatment with PACAP analogues (Figure 28). The two-way ANOVA showed significant differences in PAC1R protein levels between genotypes, with decreased levels of PAC1R in R6/1 compared to WT mice (Figure 28, B; two-way ANOVA genotype effect $F(1,52) = 25.3$, $p<0.001$). Remarkably, treatment with analogues did not induce significant changes in PAC1R protein levels (Figure 28, B; two-way ANOVA treatment effect, $F(2,52) = 0.589$, $p=0.5587$). However, Tukey's post-hoc test indicated a significant decrease in PAC1R in the hippocampus of R6/1 A1 mice compared to WT VEH mice (Figure 28, B). In conclusion, beneficial effects of PACAP analogues are not associated with the increase of PAC1R protein levels.

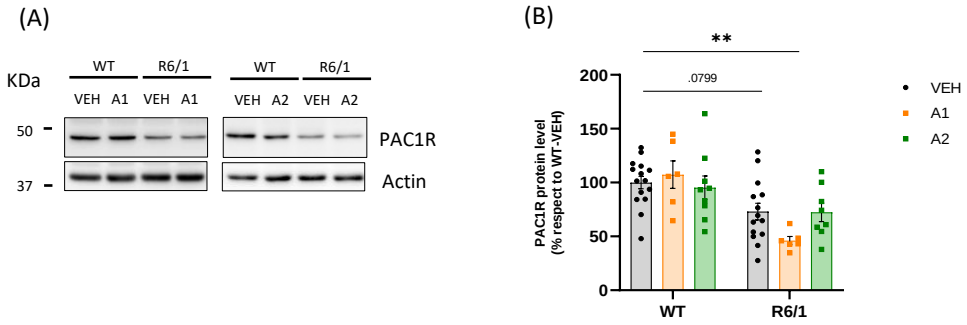
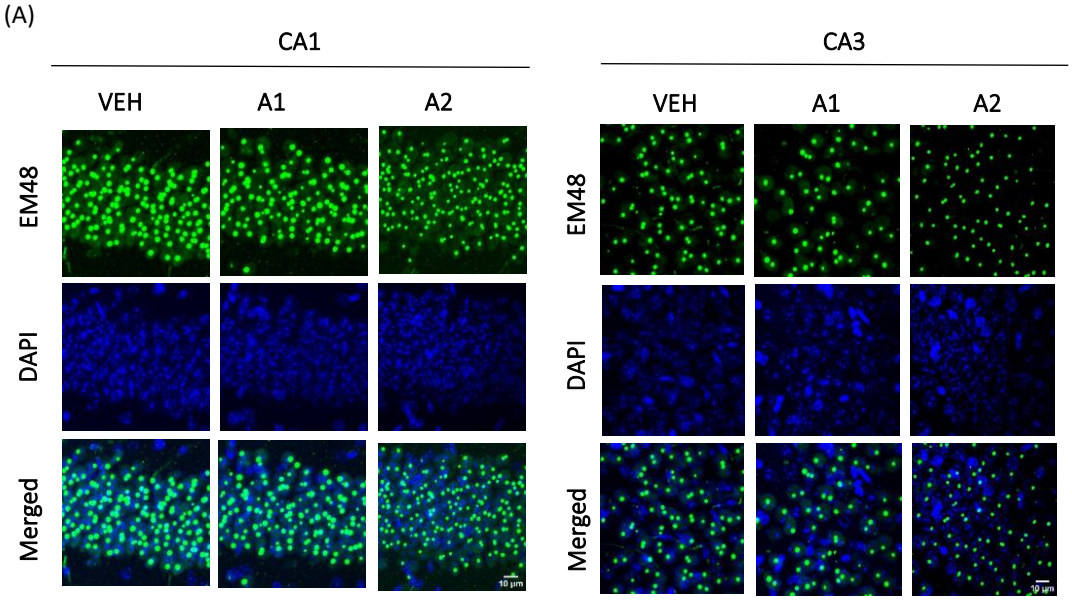


Figure 28. Intranasal administration of PACAP analogues does not increase protein levels of PAC1R. Brain samples from WT and R6/1 mice treated with PBS as vehicle (VEH), A1, or A2, were used to study hippocampal protein levels of PAC1R by western blotting. **(A)** Representative images of immunoblotting are shown. Histograms represent the densitometry quantification of **(B)** PAC1R normalized to actin for each sample and expressed as a percentage of WT VEH. Data are presented as mean ± SEM. Number of animals per group: WT VEH: 15, WT A1: 6, WT A2: 9, R6/1, VEH: 15, R6/1 A1: 6, R6/1 A2: 9. All data were analyzed using two-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. **p<0.01.

2.8. Intranuclear aggregates of mHTT are reduced in the hippocampus of R6/1 mice treated with analogues.

Finally, we studied whether A1 and A2 administration could prevent or reduce mHTT aggregation in the hippocampus of R6/1 mice (Figure 29). Interestingly, treatment with PACAP analogues had a significant effect on the number of mHTT intranuclear aggregates in all hippocampal regions analyzed (Figure 29, B-D; CA1: Welch's ANOVA W (2,8.071) = 4.665, p=0.045; CA3: one-way ANOVA F (2,20) = 4.714, p=0.0210; DG: one-way ANOVA F (2,20) = 8.424, p=0.0022) without causing any significant effect on its volume (Figure 29, E-G; CA1: one-way ANOVA F (2,20) = 1.826 , p= 0.1869; CA3: one-way ANOVA F (2,20) = 3.273, p=0.0589; DG: one-way ANOVA F (2,20) = 1.966 , p= 0.1662). Importantly, post-hoc Dunnett's test revealed that R6/1 animals treated with A1 presented reduced number of mHTT aggregates in all regions analyzed, whereas the reduction in mHTT aggregates in animals treated with A2 was only significant in the hippocampal DG (Figure 29, B-D). In conclusion, our results demonstrate that A1 can reduce or inhibit mHTT aggregation in the hippocampal CA1, DG, and CA3 of R6/1 mice, whereas A2 action on mHTT aggregates is restricted to the DG.

(A)



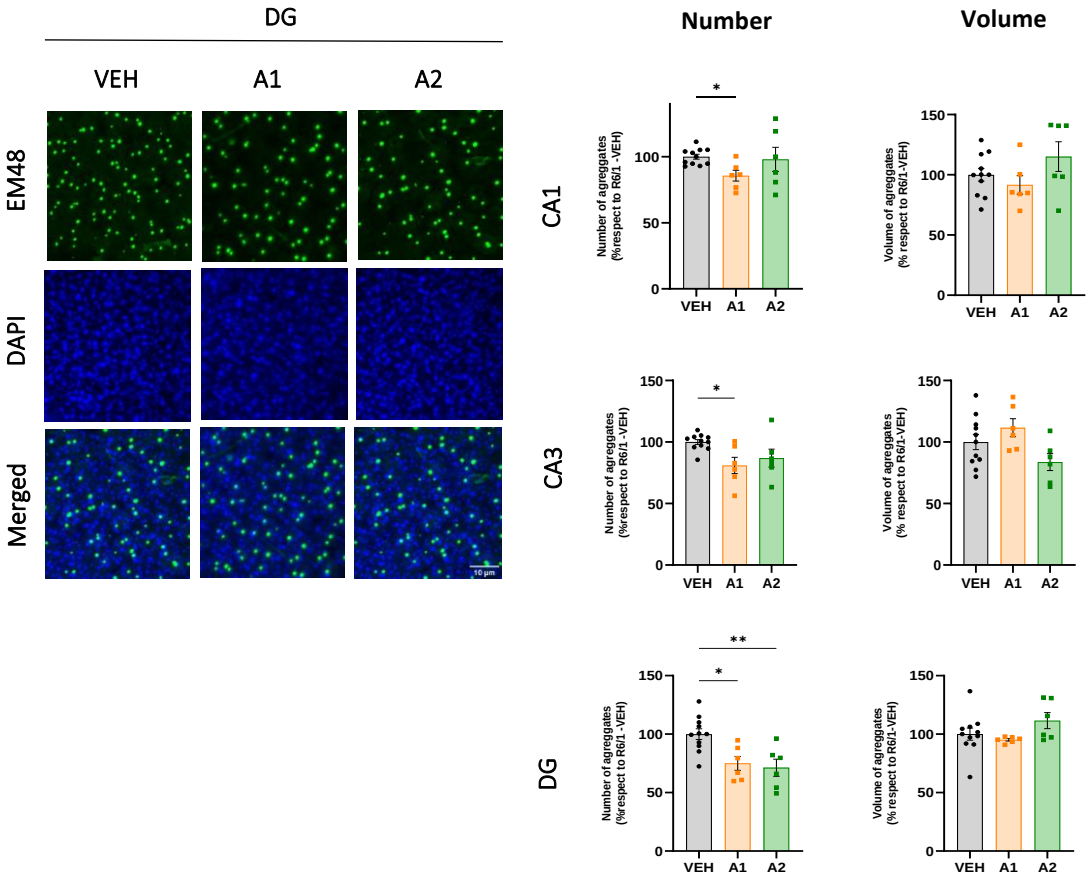


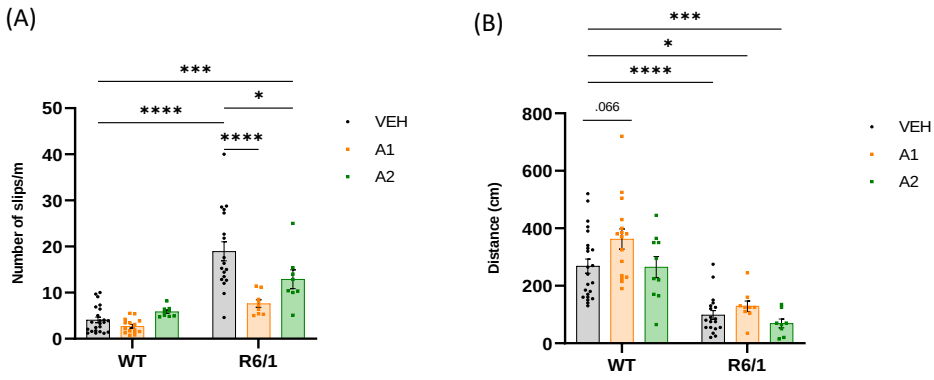
Figure 29. Treatments with A1 and A2 reduce the number of intranuclear inclusions of mHTT in the hippocampus of R6/1 treated mice. Brain samples from R6/1 mice treated with PBS as vehicle (VEH), A1 or A2, were used for immunofluorescence staining of the EM48 -positive inclusions. Z-stack confocal images were taken every 1 µm to analyze the number and volume of mHtt aggregates in the CA1, CA3 and DG hippocampal regions. Number and volume of mHTT aggregates were obtained using the 3D-object counter tool of ImageJ. The total number of mHTT aggregates was divided by the volume analyzed (area of images multiplied by the number of confocal z-stacks of 1 µm analyzed) to obtain the number of mHTT aggregates per μm^3. Finally, data was expressed as a percentage respect to R6/1-VEH mice. **(A)** Representative immunofluorescence images showing intranuclear mHTT aggregates in green and nuclei in blue in the hippocampal CA1, DG, and CA3 of VEH-, A1-, and A2- treated R6/1 mice. 2-D representative images were obtained by applying maximum intensity Z-projection (Image J software). Histograms represent the number of **(B-D)** EM48- positive inclusions per μm^3 and **(E-G)** volume of EM48-positive inclusions in the CA1, CA3, and DG expressed as a percentage of R6/1 VEH mice. Data are represented as the mean ± SEM. Number of animals per group: WT VEH: 11, WT A1: 6, WT A2: 6, R6/1 VEH: 11, R6/1 A1: 6, R6/1 A2:6. Data (E-G) was analyzed using one-way ANOVA followed by Dunnett's post-hoc test. When variances between groups were not equal (B) Welch's correction was applied. *p<0.05, **p<0.01.

3. Therapeutic potential of PACAP analogues in the striatal neuropathology and associated motor symptomatology.

3.1. Intranasal administration of PACAP analogues improves motor function in R6/1 mice.

Motor impairments observed in HD models have been related to cortico-striatal disconnection, which leads to striatal neuronal dysfunction (Fernández-García et al., 2020). Previously, we demonstrated that intranasal administration of PACAP improves motor function in R6/1 mice (Solés-Tarrés et al., 2022). Here, we investigated whether A1 and A2 could rescue the motor deficits observed in R6/1 mice. We performed a total of twelve daily intranasal administrations of PBS, A1 or A2 to WT and R6/1 mice from 18 to 20 weeks of age. From the 7th day of treatment, we subjected the animals to balance beam and rotarod tests designed to assess balance and motor coordination. In the balance beam test, we measured the number of slips per distance covered. We found that R6/1 mice presented remarkable motor impairments, with significantly more slips per meter than WT mice (Figure 30, A; two-way ANOVA genotype effect $F(1,76) = 58.95$, $p<0.0001$). Importantly, treatment with PACAP analogues had a significant effect on test performance (Figure 30, A; two-way ANOVA treatment effect, $F(2,76) = 12.26$, $p<0.0001$). Tukey's post-hoc analysis revealed that R6/1 mice treated with A1 or A2 presented significantly fewer slips per meter than R6/1 mice treated with PBS, whereas no changes were observed between vehicle- and analogues-WT-treated mice (Figure 30, A). Notably, we found that genotype and treatment also had a significant effect on the distance covered by the animals (Figure 30, B; two-way ANOVA genotype effect $F(2, 1,79) =68.57$, $p<0.0001$; two-way ANOVA treatment effect $F(2,79) = 3.703$, $p=0.0290$). Tukey's post-hoc analysis revealed that R6/1 mice, with or without treatment, walked significantly less distance than WT mice. However, A1- treated animals showed a tendency to increase the distance covered (Figure 30, B).

To further characterize the motor improvement induced by A1 and A2, we evaluated motor coordination in the rotarod test, measuring the number of falls in a minute at velocities of 12, 16, and 24 rpm. As expected, we found that R6/1 mice had a significant increase in the number of falls per minute compared to WT mice at all speeds analyzed (Figure 30, C-E; 12 rpm: two-way ANOVA genotype effect F (1,75) =28.40, p<0.0001; 16 rpm: two-way ANOVA genotype effect F (1,70) =16.21, p=0.0001; 24 rpm: two-way ANOVA genotype effect F (1,70)=37.85, p<0.0001). Intranasal treatments with analogues had a significant effect on test performance at 12 and 16 rpm (Figure 30, C-D; 12 rpm: two-way ANOVA treatment effect F (2,75) = 4.741, p=0.0115; 16 rpm: two-way ANOVA treatment effect F (2,70) =9.903, p=0.0002). Importantly, R6/1 mice treated with analogues showed improved motor coordination, as Tukey's post-hoc analysis revealed significant differences between A1- and vehicle-treated R6/1 mice at the speed of 16 rpms and between A2- and vehicle-treated R6/1 mice at speeds of 12 and 16 rpm (Figure 30, C-D). Interestingly, Tukey's post hoc test did not show significant differences between vehicle- and analogues-treated WT mice (Figure 30, C-E). Overall, we conclude that intranasal administration of A1 and A2 successfully ameliorated motor function in R6/1 mice.



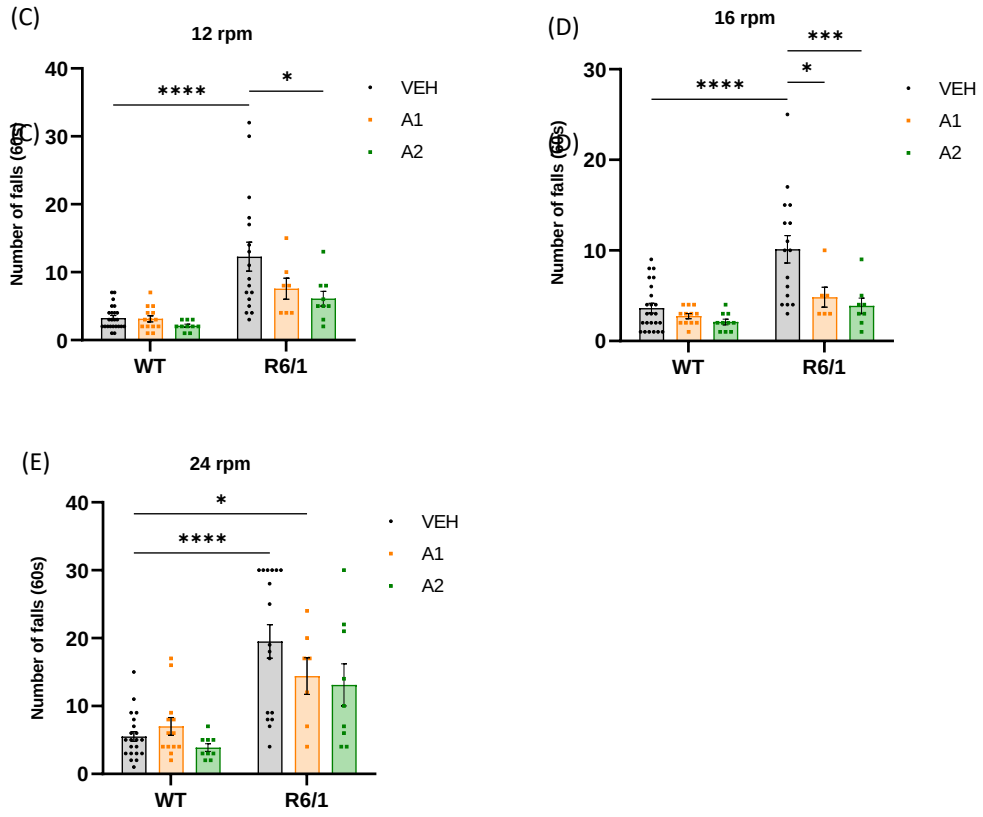


Figure 30. Treatment with PACAP analogues improves motor coordination and balance in R6/1 mice. WT and R6/1 mice were treated daily for twelve days with PBS as vehicle (VEH), A1, and A2 at 18 weeks of age. Behavioral tests were performed during the second week of treatment. In the balance beam test, we calculated **(A)** the number of slips/meters covered and **(B)** the distance travelled. In the rotarod task, we recorded the number of falls in a minute at **(C)** 12, **(D)** 16, and **(E)** 24 rpm. Data are presented as the mean ± SEM. Number of animals per group: WT VEH: 23-25, WT A1: 13-15, WT A2: 10, R6/1 VEH: 17-19, R6/1 A1: 7-9, R6/1 A2:9. All data were analyzed by using two-way ANOVA followed by Tukey's as post-hoc test. *p<0.05, ***p<0.001, ****p<0.001.

3.2. Intranasal administration of A1 protects medium spiny neurons in the striatum of R6/1 mice.

Striatal volume loss occurs in the physiopathology of HD because of extensive loss of medium spiny neurons (MSNs) (Vonsattel et al., 1985). To investigate the effect of

treatment with PACAP analogues on striatal volume, we performed Nissl staining (Figure 31, A-B). Our results showed a significant reduction in striatal volume of R6/1 mice compared to WT mice, with no changes after treatment with peptides (Figure 31, B; two-way ANOVA genotype effect $F(1,37) = 128.2$, $p<0.001$; two-way ANOVA treatment effect $F(2,37) = 0.9612$, $p=0.392$). Then, we studied the MSNs population using Dopamine- and cAMP-regulated phosphoprotein 32 kDa (DARPP-32) staining (Figure 31, C-F). The results showed a significant reduction in the number of DARPP-32 positive cells in the striatum of R6/1 mice compared with WT mice (Figure 31, D; two-way ANOVA genotype $F(1,38) = 47.8$, $p=0<0.0001$), with significant changes between treatments (Figure 31, D; two-way ANOVA treatment $F(2,38) = 9.36$, $p=0.0005$). Post-hoc Tukey's test did not show significant differences between R6/1 mice treated with peptides or vehicle, but R6/1-A1 animals did not show significant differences from WT-VEH, indicating partial recovery of the number of DARPP-32 positive cells (Figure 31, D). We then studied the morphology of DARPP-32 positive neurons by measuring their cross-transversal area. We found that the soma of DARPP-32 positive cells in the striatum of R6/1 mice was smaller than that of WT mice (Figure 31, E; two-way ANOVA genotype $F(2,282) = 14.2$, $p=0.0002$). Importantly, we found treatments had a significant effect in the size of soma (Figure 31, E; two-way ANOVA treatment effect, $F(2,282) = 9.41$, $p=0.0001$). Specifically, R6/1 mice treated with A1 exhibited a significantly increased cross-transversal area (Figure 31, E). Finally, we analyzed the intensity of DARPP-32 immunofluorescence staining to estimate DARPP-32 expression. We observed that the intensity of DARPP-32 was reduced in R6/1 mice compared to that in WT mice, but we did not detect significant changes between treatments (Figure 31, F; two-way ANOVA genotype effect $F(2,279) = 148$, $p<0.0001$, two-way ANOVA treatment effect $F(2,279) = 2.52$, $p=0.0825$). In summary, we conclude that intranasal administration of A1 protects MSNs of R6/1 mice.

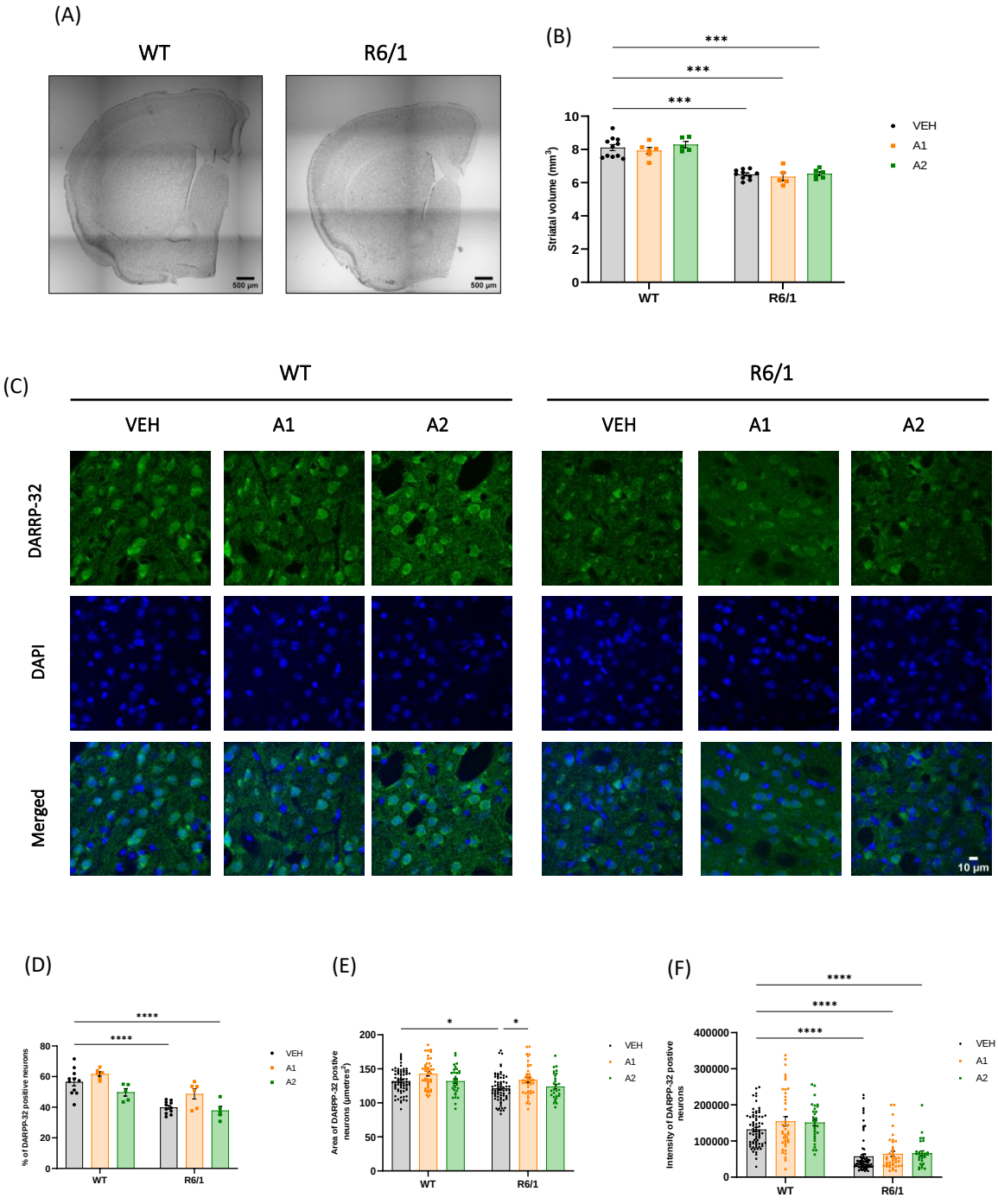

(A)
WT
R6/1
500 µm
500 µm
(B)
***
***
***
Striatal volume (mm³)
10
8
6
4
2
0
WT
R6/1
VEH
A1
A2
(C)
WT
R6/1
VEH
A1
A2
VEH
A1
A2
DARRP-32
DAPI
Merged
10 µm
(D)
****
****
% of DARPP-32 positive neurons
80
60
40
20
0
WT
R6/1
VEH
A1
A2
(E)
*
*
Area of DARPP-32 positive neurons (µmetre²)
200
150
100
50
0
WT
R6/1
VEH
A1
A2
(F)
****
****
****
Intensity of DARPP-32 positive neurons
400000
300000
200000
100000
0
WT
R6/1
VEH
A1
A2

Figure 31. Treatment with A1 protects DARPP-32 positive neurons in R6/1 mice. Brain samples from WT and R6/1 mice treated with PBS as vehicle (VEH), A1, or A2 were used for Nissl staining and immunofluorescence staining against DARPP-32. Bright-field images of Nissl-stained brains were captured, and striatal volume estimations were performed using the Cavalieri method. **(A)** Representative images showing Nissl-stained brains of VEH- WT and R6/1 mice. **(B)** Histograms represent striatal volumes obtained in mm^3. Confocal images were obtained to analyze the different parameters of DARPP-32 positive neuronal population. **(C)** Representative immunofluorescence images showing DARPP-32 positive neurons in green and nuclei in blue in VEH-, A1-, and A2- treated WT and R6/1 mice. Histograms represent **(D)** the percentage of DARPP-32 positive cells, **(E)** the cross-transverse area of DARPP-32 positive neuronal cells, and **(F)** the intensity of fluorescent staining in DARPP-32 positive cells. Data are represented as the mean ± SEM. Number of animals per group in B-C: WT VEH: 11, WT A1: 6, WT A2: 5, R6/1, VEH: 11, R6/1 A1: 5-6, R6/1 A2: 5-6. Number of DARPP-32 positive neurons analyzed in D-E: WT VEH:72 neurons from 11 mice, WT A1:42 neurons from 6 mice, WT A2:31 neurons from 5 mice, R6/1 VEH:72 neurons from 11 mice, R6/1 A1:42 neurons from 6 mice, and R6/1 A2:30 neurons from 5 mice. Data were analyzed using two-way ANOVA followed by Tukey's post-hoc test. *p<0.05, ***p<0.001, ****p<0.0001.

3.3. Beneficial effects of PACAP analogues are not associated with changes in striatal protein levels of BDNF and its receptors, TrkB and P75NTR.

Deficits in BDNF levels and reduced BDNF signaling have been related to neuronal dysfunction and degeneration of MSNs in HD. As we previously showed that PACAP increases BDNF protein levels, we studied whether PACAP analogues promote the expression of BDNF or change the expression of its receptors, TrkB and p75NTR, in the striatum of R6/1 mice (Figure 32, A-D). Unexpectedly, we did not find a significant effect of genotype on the protein levels of any protein studied (Figure 32, A-D; BDNF: two-way ANOVA genotype $F(1,42) = 1.10$, $p=0.3000$; TrkB: two-way ANOVA genotype $F(1,40) =0.116$, $p=0.7349$; p75NTR: two-way ANOVA genotype $F(1,41) = 0.500$, $p=0.4834$). In addition, treatment with A1 or A2 did not induce significant changes in the expression of these proteins (Figure 32, A-D; BDNF: two-way ANOVA treatment $F(2,42) = 1.52$, $p=0.2315$; TrkB: two-way ANOVA treatment $F(2,40) =2.77$, $p=0.0749$; p75NTR: two-way ANOVA treatment $F(2,41) = 1.45$, $p=0.2470$). Thus, our results

indicate that intranasal administration of PACAP analogues do not alter protein levels of BDNF and its receptors.

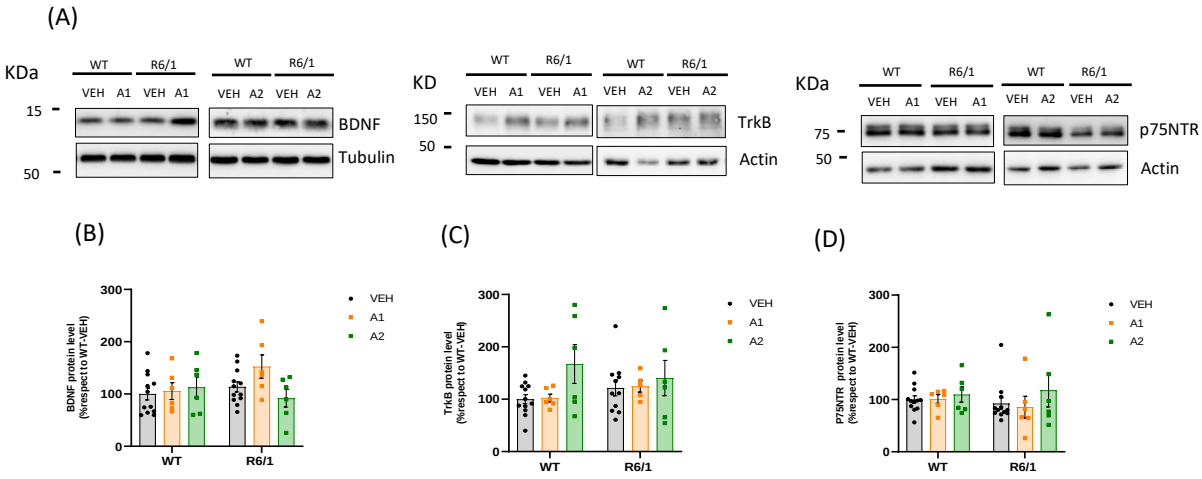


Figure 32. Treatment with PACAP analogues did not change the striatal protein levels of BDNF and its receptors TrkB and p75NTR. Brain samples from WT and R6/1 mice treated with PBS as vehicle (VEH), A1, or A2, were used to study striatal protein levels of BDNF, TrkB, and p75NTR by western blotting. **(A)** Representative images of immunoblotting for BDNF, TrkB, and p75NTR in the striatum of VEH-, A1-, and A2- treated WT and R6/1 mice. Histograms represent the densitometry quantification of **(B)** BDNF, **(C)** TrkB and **(D)** p75NTR normalized to tubulin or actin for each sample and expressed as a percentage of WT VEH. Data are presented as mean ± SEM. Number of animals per group: WT VEH: 12, WT A1: 6, WT A2: 6, R6/1, VEH: 12, R6/1 A1: 5-6, R6/1 A2:6. All data were analyzed using two-way analysis of variance (ANOVA), followed by Tukey's post-hoc test.

3.4. Intranasal administration of A1 increases the activation of pro-survival signaling pathway Akt in the striatum of R6/1 mice.

We studied if the administration of PACAP analogues could activate proteins related to neurotrophic functions as ERK1/2, Akt and phospholipase C gamma 1 (PLCγ1) in the striatum of R6/1 mice (Figure 33). Our results indicate that there was a significative genotype effect in the activation of all the three pathways analysed (Figure 33, A-D; pERK 1/2: two-way ANOVA genotype F (1,42) = 4.20, p=0.0467; pAkt: two-way ANOVA genotype F (1,41) =19.4, p<0.001; pPLCγ1: two-way ANOVA genotype F (1,49) = 0.500, p=0.0270). Importantly, two-way ANOVA of pAkt protein levels showed a genotype-

treatment interaction (Figure 33, C; two-way ANOVA interaction F (2,41) =5.54, p=0.0074). In addition, Tukey's post hoc test indicated significant differences between R6/1 A1 and R6/1 VEH, R6/1 A2, and WT VEH mice, indicating that A1 administration increases the striatal pAKT protein levels in R6/1 mice (Figure 33, B). Regarding to pERK1/2 and pPLCγ1 protein levels, we did not find significant differences after treatment with PACAP analogues (Figure 33, B and D; pERK1/2: two-way ANOVA treatment F (2,42) = 1.52, p=0.9661, two-way ANOVA interaction F (2,42) = 2.62, p=0.0845; pPLCγ1: two-way ANOVA treatment F (2,49) = 1.45, p=0.1816; two-way ANOVA interaction F (2,49) = 0.478, p=0.1319). Altogether our results indicate that only treatment with A1 induced the activation of Akt in the striatum of R6/1 mice.

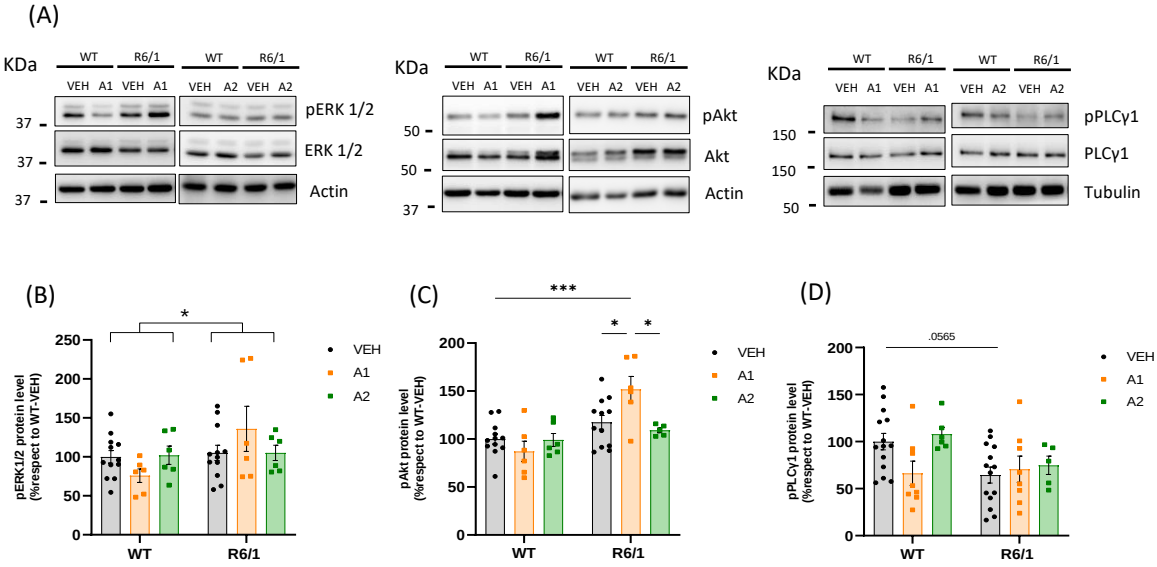
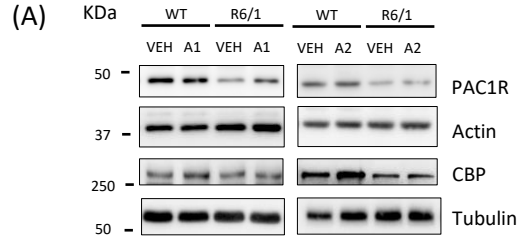


Figure 33. Treatment with A1 increases striatal protein levels of phosphorylated Akt. Brain samples from WT and R6/1 mice treated with PBS as vehicle (VEH), A1, or A2, were used to study striatal protein levels of pERK1/2, pAkt (Ser473), and pPLCγ1 (Tyr783) by western blotting. **(A)** Representative images of immunoblotting for pERK1/2, pAkt, and pPLCγ1 in the striatum of VEH-, A1-, and A2- treated WT and R6/1 mice. Histograms represent the densitometry quantification of **(B)** pERK1/2, **(C)** pAkt, and **(D)** pPLCγ1 normalized to total protein levels of ERK1/2, Akt and PLC for each sample and expressed as a percentage of WT VEH. Data are presented as mean ± SEM. Number of animals per group: WT VEH: 11-14, WT A1: 6-7, WT A2: 5-6, R6/1, VEH: 11-15, R6/1 A1: 6-8, R6/1 A2:5-6. All data were analyzed using two-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. *p<0.05, ***p<0.001.

3.5. Beneficial effects of A1 are associated with the partial recovery of striatal PAC1R protein levels.

We previously reported that the beneficial effects of PACAP in the striatum were associated with a recovery of its specific receptor PAC1R and an increase in the CREB-coactivator CBP. Therefore, we investigated the effect of PACAP analogues treatment on the expression of these proteins (Figure 34). Our results confirmed the decreased levels of PAC1R in the striatum of R6/1 mice (Figure 34, B; two-way ANOVA genotype $F(1,42) = 42.7$, $p<0.001$). In addition, ANOVA indicated that treatment had a significant effect on PAC1R levels (Figure 34, B; two-way ANOVA treatment $F(2,42) = 7.37$, $p=0.0018$). Although we did not find significant differences between R6/1 VEH and R6/1 A1, Tukey's post-hoc test also showed no significant differences between WT VEH and R6/1 A1 mice, indicating a partial recovery of PAC1R protein levels in these animals (Figure 34, B). Regarding CBP, we found significant differences between genotypes (Figure 34, C; two-way ANOVA genotype $F(1,42) = 6.84$, $p=0.0124$), but treatment with PACAP analogues did not induce significant changes (Figure 34, C; two-way ANOVA treatment $F(2,42) = 1.37$, $p=0.2647$).



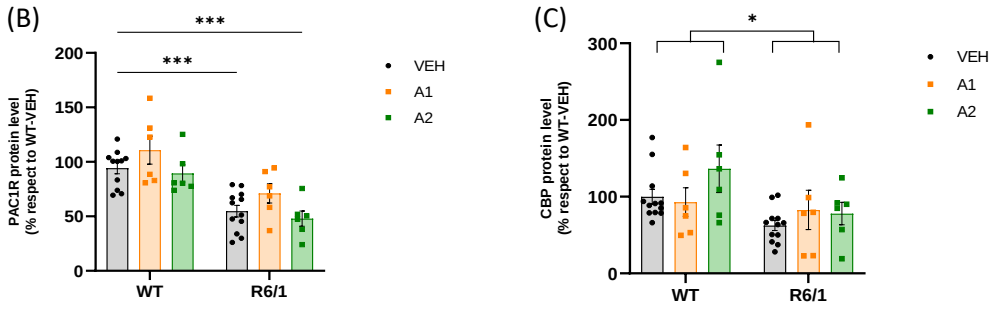


Figure 34. Treatment with PACAP analogues does not increase PAC1R neither CBP protein levels in the striatum of R6/1 mice. Brain samples from WT and R6/1 mice treated with PBS as vehicle (VEH), A1, or A2, were used to study striatal protein levels of PAC1R and CBP by western blotting. (A) Representative images of immunoblotting for PAC1R and CBP in the striatum of VEH-, A1-, and A2- treated WT and R6/1 mice. Histograms represent the densitometry quantification of **(B)** PAC1R and **(C)** CBP normalized to actin or tubulin for each sample and expressed as percentage of WT. Data are presented as the mean ± SEM. Number of animals per group: WT VEH: 11-12, WT A1: 6, WT A2: 6, R6/1, VEH: 11-12, R6/1 A1: 5-6, R6/1 A2: 6. All data were analyzed by using two-way ANOVA followed by Tukey's post-hoc test. *p<0.05, ***p<0.001.

3.6. Intranasal administration of A1 reduces the number of intranuclear inclusions of mHTT in the striatum of R6/1 treated mice.

We then studied whether A1 and A2 administration could prevent or reduce mHTT aggregation in the striatum alike we showed in the hippocampus. Thus, we measured the number and volume of mHTT aggregates in dorsolateral striatum of R6/1 mice (Figure 35). Our results showed significative differences between treatments on the number of mHTT intranuclear aggregates in striatum (Figure 35, B; Kruskall Wallis statistic = 9.22, p= 0.0044) with no significant differences on its volume (Figure 35, C; One-way ANOVA F (2,19) = 2.963, p=0.0758). Remarkably, the post-hoc Dunn's test revealed that R6/1 animals treated with A1 presented a significant reduction in the number of mHTT intranuclear inclusions (Figure 35, B). Hence, only A1 has the capacity to reduce or inhibit the mHTT aggregation in the striatum of R6/1 mice.

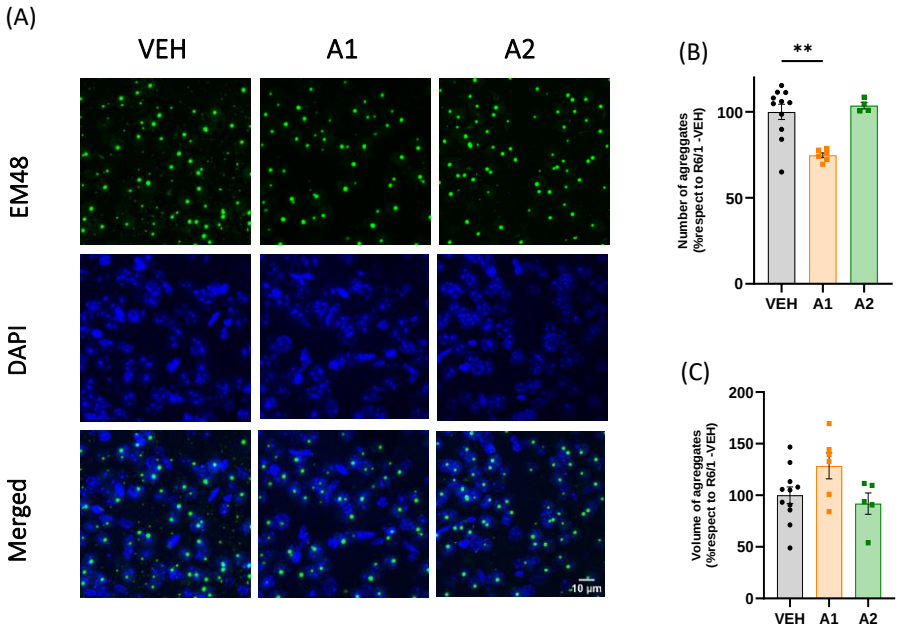


Figure 35. R6/1 animals treated with A1 showed reduced number of striatal intranuclear inclusions of mHTT. Brain samples from R6/1 mice treated with PBS as vehicle (VEH), A1 or A2, were used for immunofluorescence staining of the EM48 -positive inclusions. Z-stack confocal images were taken every 1 µm to analyze the number and volume of mHtt aggregates in dorsolateral striatum. Number and volume of mHTT aggregates were obtained using the 3D-object counter tool of ImageJ. The total number of mHTT aggregates was divided by the volume analyzed (area of images multiplied by the number of confocal Z-stacks of 1 µm analyzed) to obtain the number of mHTT aggregates per μm^3. Finally, data was expressed as a percentage respect to R6/1-VEH mice. **(A)** Representative immunofluorescence images showing intranuclear mHTT aggregates in green and nuclei in blue in the striatum of VEH-, A1-, and A2- treated R6/1 mice. 2-D representative images were obtained by applying maximum intensity Z-projection (Image J software). Histograms represent the quantification of the **(B)** number of EM48- positive inclusions per μm^3 and **(C)** volume of EM48-positive inclusions expressed as a percentage of R6/1 VEH mice. Data are represented as the mean ± SEM. Number of animals per group: WT VEH: 11, WT A1: 6, WT A2: 5, R6/1 VEH: 11, R6/1 A1: 6, R6/1 A2:5. Parametrical data (C) were analyzed by using one ANOVA followed by Dunnet's as post-hoc test, while non-parametric data (B) were analyzed by using Kruskall Wallis test followed by Dunn's as post-hoc test. **p<0.01.

CHAPTER V: DISCUSSION

Huntington's disease (HD) is a devastating disorder caused by the expression of mutant huntingtin (mHTT). Currently, there is no effective treatment to prevent the natural progression of the disease. HD patients suffer from a triad of symptoms, including motor discoordination, cognitive impairment, and psychiatric disturbances. In this study, we focused on motor and cognitive alterations, which are particularly associated with the dysfunction and degeneration of the striatum and hippocampus. Specifically, loss of striatal neurons is a key feature in the development and progression of motor discoordination (Slow et al., 2003; Guo et al., 2012a), whereas hippocampal dysfunction has been associated with cognitive deficits in HD (Giralt et al., 2012b; Harris et al., 2019). In both cases, alterations in molecular mechanisms, such as transcriptional dysregulation, neurotrophic deprivation, or alteration of glutamate transmission, mediate neuronal dysfunction, leading to neuronal death. Therefore, it has been suggested that compounds that can promote synaptic plasticity and neuronal survival could be interesting therapeutic agents for HD.

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide that is widely distributed throughout the central nervous system (CNS) with the capacity to promote synaptic plasticity and protect against neuronal apoptosis through activation of its receptors, PAC1R, VPAC1R, and VPAC2R, in different neuronal populations (Vaudry et al., 2009). Recently, our laboratory demonstrated that intranasal administration of PACAP improved cognitive and motor deficits in a transgenic mouse model of HD (Cabezas-Llobet et al., 2018; Solés-Tarrés et al., 2022) by enhancing synaptic function. However, the protective effects of PACAP and the role of its receptors against mHTT-mediated toxicity were not entirely explored. In this thesis, we show that PACAP protects against mHTT-induced toxicity and that PAC1R plays a key role in this action.

Similar to other molecules with therapeutic effects, PACAP has some pharmacological limitations. First, the bioavailability of PACAP is very low. In addition, PACAP administration has been reported to cause side effects, such as hypertension or

tachycardia, due to the peripheral activation of VPACR (Bourgault et al., 2009a). To overcome these two major limitations, metabolically stable analogues of PACAP that display a higher preference for PAC1R have been proposed. In the present study, we demonstrate for the first time that two novel analogues of PACAP improve cognitive and motor functions in a HD mouse model. Moreover, we describe some of their mechanisms of action in the hippocampus and the striatum.

PAC1R plays a key role in PACAP-induced neuroprotection against mHTT-mediated toxicity.

PAC1R is the specific receptor of PACAP and has been suggested to be the main effector of PACAP neuroprotective actions. To explore the therapeutic potential of PAC1R activation against mHTT toxicity, we used the STHdh cellular model of HD, optimal to characterize the molecular alterations caused by the presence of mHTT. We found reduced protein levels of PAC1R in Q111 cells compared to those in Q7 cells. The reduction in PAC1R is in line with our previous results, since we have described this decrease in the striatum and motor cortex of R6/1 mice, as well as the hippocampus (Cabezas-Llobet et al., 2018; Solés-Tarrés et al., 2022). It has also been found a downregulation of PAC1R in the caudate and putamen of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced macaque model of Parkinson's disease (PD) (Feher et al., 2018), and similar results have been obtained in the motor cortex of patients with amyotrophic lateral sclerosis (ALS) (Bonaventura et al., 2018). Regarding VPACR, we found a reduction of VPAC1R while VPAC2R protein levels remained unaltered in Q111 cells. We previously observed reduced levels of VPAC2R in the striatum of R6/1 mice, but at very late stages of the disease (Solés-Tarrés et al., 2022). In addition, there is a reduction of all receptors in the hippocampus of R6/1 and Knock-in mice from 12 weeks and 17 months, respectively (Cabezas-Llobet et al., 2018). Thus, we can conclude that reduced protein levels of PAC1R is a molecular alteration occurring in different HD models and can be accompanied by a reduction of VPACR protein levels.

Importantly, these alterations may be part of the neuropathological process, as has been proposed in other neurodegenerative disorders.

One of the most described neuroprotective actions of PACAP is its anti-apoptotic effect. Importantly, we found that addition of PACAP prevents apoptosis in Q111 cells by the reduction of cleaved caspase-3 levels. It has been described that PACAP protects against oxidative stress and apoptotic cell death by inhibiting caspase-3 activity in rat cerebellar granule cells (Vaudry et al., 2000, 2002; Falluel-Morel et al., 2004) and retinal ganglion cells (Ye et al., 2019). Similarly, in PC12 cells with β-amyloid- or rotenone-induced neurotoxicity, the neuroprotective activity of PACAP is mediated by caspase-3 inhibition (Onoue et al., 2002a; Wang et al., 2005). Importantly, some authors have suggested that PAC1R is the main effector of PACAP-induced antiapoptotic effects (Vaudry et al., 2000; Onoue et al., 2002a; Campard et al., 2009; Seaborn et al., 2011). In addition, PACAP can enhance the expression of its specific receptor PAC1R (Shintani et al., 2005; Rat et al., 2011; Georg et al., 2016). Accordingly, we previously reported that administration of PACAP enhanced PAC1R expression in the hippocampus and striatum of R6/1 mice (Cabezas-Llobet et al., 2018; Solés-Tarrés et al., 2022). Thus, we analyzed PAC1R protein levels after PACAP treatment in striatal cells. Our results demonstrated that the beneficial effects of PACAP were not associated with the upregulation of PAC1R expression in Q111 cells, suggesting that activation of PACAP receptors was sufficient to promote pro-survival effects in this model.

To know the contribution of PACAP receptors in PACAP-mediated protective effect, we used vasoactive intestinal polypeptide (VIP), which exhibits equal affinity for VPAC1R and VPAC2R, and thousand-fold lower affinity for PAC1R compared to PACAP. We found that treatment with VIP also inhibited caspase-3 activation, indicating that VPACR can also initiate anti-apoptotic cascade signaling in Q111 cells. The ability of VIP to inhibit the activity of caspase-3 has been described previously in cerebellar granule cells but treated with higher concentrations in comparison to PACAP (Vaudry et al.,

2000; Onoue et al., 2002a). It has also been described that VIP can protect against dopamine and 6-hydroxydopamine (6-OHDA) toxicity in PC12 and neuroblastoma cells and against 6-OHDA toxicity in cerebellar granule cells by preventing the oxidative stress (Offen et al., 2000). This is consistent with the findings of Tunçel and colleagues in a 6-OHDA murine model, in which VIP administration protected neuronal tissues from oxidative stress and apoptosis by reducing lipid peroxidation and DNA fragmentation (Tunçel et al., 2012). Oxidative stress has been reported in Q111 cells due to mitochondrial dysfunction (Jin et al., 2013). Therefore, it is temptative to suggest that VPACR activation in Q111 cells could protect against oxidative stress, resulting in the inhibition of caspase-3. Overall, we propose that it is likely that all PACAP receptors participate in PACAP-mediated anti-apoptotic action, initiating different mechanisms that converge in the inhibition of caspase-3.

PACAP receptors can activate extracellular signal-regulated kinases 1/2 (ERK1/2) and Akt (Dickson and Finlayson, 2009), the two main pathways regulating cell survival and neurotrophic effects (Rai et al., 2019). As altered expression and activity of ERK1/2 and Akt has been found in human HD samples and in multiple models of HD, including Q111 cells (Humbert et al., 2002; Colin et al., 2005; Apostol et al., 2006; Ginés et al., 2010; Roze et al., 2011; Bodai and Marsh, 2012), we explored the effect of PACAP and VIP treatments on phosphorylation of ERK1/2 and Akt. Interestingly, our results showed that PACAP, and not VIP, induces a rapid and transient activation of ERK1/2 and Akt in STHdh cells, indicating that PAC1R is necessary for their activation. Additionally, we described by using specific inhibitors, that ERK1/2 and Akt activation play a key role in PACAP-mediated inhibition of caspase-3 in Q111 cells. Importantly, it has been described that PAC1R mediates the inhibition of caspase-3 by the activation of ERK1/2, which enhances the expression of c-fos and B-cell lymphoma 2 (Bcl-2), finally leading to the inhibition of the release of cytochrome c from the mitochondria (Dejda et al., 2008; Seaborn et al., 2011). In addition several lines of evidence indicate that ERK1/2 is involved in a wide variety of PACAP-PAC1R-generated responses, such as the

induction of PACAP gene expression in human neuroblastoma NB-1 cells (Georg et al., 2016), differentiation of PC12 cells to sympathetic-like neurons (Barrie et al., 1997), protection of cerebellar granule cells from apoptosis (Villalba et al., 1997), and induction of neuritogenesis in Neuroscreen-1 (NS-1) cells, a subtype of PC12 cells (Emery and Eiden, 2012). Similarly, the activation of PAC1R has been found to increase phosphorylated Akt (pAkt) in the primary sympathetic neuronal cultures promoting survival (May et al., 2010).

Although our results showed the involvement of ERK1/2 and Akt in the PACAP-PAC1R-mediated neuroprotection against mHTT toxicity, we cannot discard the contribution of other survival pathways. PACAP receptors are coupled to G proteins, they can promote the activation of the adenylate cyclase (AC)/ protein kinase A (PKA) and phospholipase C (PLC) / protein kinase C (PKC) pathways as well as the release of Ca^{2+} from the endoplasmic reticulum. Importantly, the contribution of these pathways to anti-apoptotic action has been shown in different studies, and it is suggested to differ depending on the neuronal cell type and the neurotoxic agent. For instance, in PC12 cells with rotenone- or prion protein fragment-induced neurotoxicity, PACAP-mediated inhibition of caspase-3 is facilitated by the activation of both ERK1/2 and PKA (Onoue et al., 2002b; Wang et al., 2005). In cerebellar granule cells, PACAP inhibits caspase-3 activity via PKA- and PKC-dependent mechanisms but without the participation of ERK1/2 (Vaudry et al., 2000). In early experimental diabetic retinopathy, PACAP promoted neuronal survival and inhibited caspase-3 through the activation of ERK1/2, Akt, and PKC (Szabadfi et al., 2014). Thus, based on previous observations and our results, it is temptative to suggest that although the activation of ERK1/2 and Akt by PACAP-PAC1R seems to be a key survival mechanism to fight mHHT-induced cell death, other pathways not explored in this thesis, such as the PKA and PKC pathways, could contribute to PACAP-mediated survival in Q111 cells.

In this line, the fact that VIP could reduce cleaved caspase-3 protein levels without inducing ERK1/2 and Akt activation, indicates that the effect of VPACR may be

associated with the activation of these pathways. Supporting this hypothesis, it has been found that VIP protects against apoptosis induced by oxidative stress phosphorylating Bcl-2 family member BAD in a PKA-dependent matter (Sastry et al., 2006). In addition, VPAC1R activation protects against neuronal cell death through activation of the cAMP/PKA pathway (Delgado et al., 2008). Overall, these results support the idea that different pathways linked to all PACAP receptors are likely to participate together in the anti-apoptotic effect induced by PACAP. Specifically, while PAC1R activates the pro-survival ERK1/2 and Akt pathways, VPACR likely activate PKA, which is mainly associated with the protection against oxidative stress.

Protective actions of PACAP receptors have been associated with the transcriptional upregulation of neuroprotective genes. Importantly, transcriptional dysregulation is a characteristic pathological process of HD, and a reduction of immediate early genes (iEGs) involved in neuronal activity, plasticity, and survival (Zhang et al., 2002; West and Greenberg, 2011; Keilani et al., 2012), such as c-fos and egr1, has been observed in R6/1 mouse model (Desplats et al., 2006; Anglada-Huguet et al., 2016a; Cabezas-Llobet et al., 2018). Thus, we studied whether PACAP and VIP could promote this effect in Q111 cells. Interestingly, we found that PACAP treatment increased the expression of c-fos and egr1. As no effect was observed in the presence of VIP, we suggest that the PACAP-mediated upregulation of c-fos and egr1 is related to PAC1R activation. These findings are supported by other studies in which PACAP has been shown to stimulate the expression of a wide variety of iEGs in neurons (Vaudry et al., 1998; Ravni et al., 2006, 2008). One of the proteins most studied for its role in the transcriptional dysfunction that occurs in HD is the coactivator named cAMP-response element-binding protein (CREB) binding protein (CBP). The presence of mHTT causes a decrease in CBP levels and activity, resulting in the inhibition of CREB target genes, including the brain neurotrophic factor (BDNF) (Steffan et al., 2000; Nucifora et al., 2001; Sugars et al., 2004). Importantly, in the pathology of HD, loss of BDNF has been found in HD patients as well as in several animal models that contribute to the onset and severity

of symptoms (Duan et al., 2001; Zuccato et al., 2001, 2005; Luthi-Carter et al., 2002; Gines et al., 2003b; Hermel et al., 2004; Spires et al., 2004b; Lynch et al., 2007; Gharami et al., 2008; Diekmann et al., 2009; Giralt et al., 2009, 2011a; Simmons et al., 2011; Cabezas-Llobet et al., 2018). Our results showed that PACAP treatment increased protein levels of both CBP and BDNF in Q111 cells. Importantly, similar to the results obtained for iEGS genes, these effects were not shared with VIP, indicating that PAC1R plays an essential role in this action. We previously demonstrated that intranasal administration of PACAP restored CBP and BDNF protein levels in the hippocampus and striatum of R6/1 mice (Cabezas-Llobet et al., 2018; Solés-Tarrés et al., 2022). Thus, we propose that PACAP-PAC1R signaling could increase CBP levels and, consequently, CREB-dependent transcription, finally leading to the recovery of BDNF protein levels. Accordingly, PAC1R activation has been found to enhance CREB phosphorylation and BDNF expression in different *in vitro* and *in vivo* neurodegenerative models. (Baxter et al., 2011; Emery and Eiden, 2012; Brown et al., 2013). In addition, PACAP administration increased BDNF expression in different murine models (Rat et al., 2011; Ladjimi et al., 2019), as well as in cultured neurons (Frechilla et al., 2001; Shintani et al., 2005). Taken together, our results indicate that PACAP induces changes in transcription and enhances the expression of neurotrophic proteins that are altered in HD through activation of the PAC1R receptor.

In summary, we provide evidence of that although all PACAP receptors may participate in PACAP-promoted antiapoptotic action in HD, the specific activation of PAC1R plays a key role promoting the activity of pro-survival pathways, and the expression of neuroprotective and neurotrophic proteins altered in the context of mHTT expression (Figure 36). Therefore, we suggest that molecules with enhanced affinity to PAC1R could be attractive therapeutic agents to treat HD.

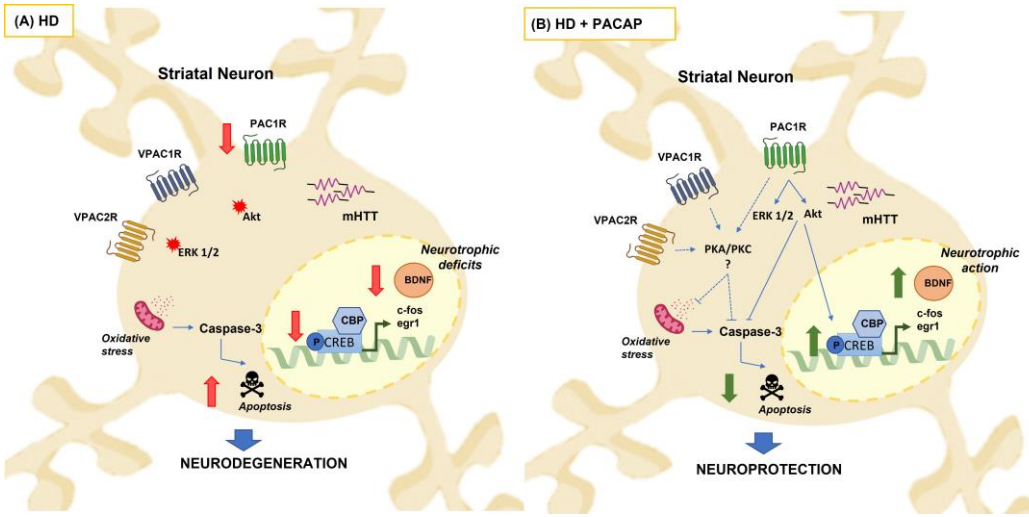


Figure 36: Involvement of PACAP receptors in PACAP-induced neuroprotection. Schematic representation of the mechanisms proposed by which the activation of PACAP receptors protects striatal neurons from mutant huntingtin (mHTT) toxicity. (A) Striatal neurons expressing mHTT exhibit diminished protein levels of PAC1R, an alteration in activity of pro-survival pathways such as extracellular signal-regulated kinases 1/2 (ERK1/2) and Akt, neurotrophic deficits, and increased levels of caspase-3 (B) Treatment with PACAP and VIP prevents from mHTT-induced apoptosis indicating that PACAP receptors exert a protective effect. The activation of ERK1/2 and Akt is only related to PACAP-mediated neuroprotective action, indicating a specific role of PAC1R in the activation of these pro-survival pathways. However, other pathways likely linked to both PAC1R and VPACR, such as protein kinase A (PKA) and protein kinase C (PKC), also participate in this neuroprotection, maybe preventing from oxidative stress. Additionally, the differences between PACAP and VIP treatments indicate that enhanced expression of c-fos, egr1, cAMP response element-binding protein (CREB)-binding protein (CBP), and brain-derived neurotrophic factor (BDNF) is related to PAC1R activation. Discontinuity in the arrows indicate molecular mechanisms proposed in this discussion based on previous studies.

PACAP analogues improve spatial-memory function in R6/1 mice model of HD enhancing the hippocampal neuroplasticity.

Cognitive dysfunction occurs early in HD patients as well as in mouse models of HD. Accumulating evidence suggests that hippocampal dysfunction contributes to such impairments (Giralt et al., 2012b). Accordingly, significant deficits in spatial memory tasks, which are dependent on hippocampal function, have been described in both

people with initial HD (Glikmann-Johnston et al., 2019, 2021; Harris et al., 2019) and in different mouse models of HD in the early stages (Lione et al., 1999; Lüesse et al., 2001; Nithianantharajah et al., 2008; Brooks et al., 2012a; Giralt et al., 2012b).

According to previous studies (Nithianantharajah et al., 2008; Cabezas-Llobet et al., 2018), we found that R6/1 mice display deficits in hippocampal-dependent spatial memory tasks at 14 weeks of age. Importantly, our results demonstrated that intranasal administration of both analogues rescued spatial memory deficits evaluated by the T-MAZE alternation test with an inter-trial interval (ITI) of 1 hour. Moreover, A2 was able to improve the spatial memory deficits of R6/1 mice evaluated by the novel object localization test (NOLT) with an ITI of 24 hours. Activation of PACAP receptors to improve cognitive function has been demonstrated in different rodent models of neurodegenerative diseases. In MPTP-treated mice, PACAP improved spatial working memory and learning (Deguil et al., 2009) while in APP[V717I]-transgenic mice long-term treatment with PACAP improved recognition memory deficits (Rat et al., 2011). In addition, our group previously demonstrated that intranasal treatment with PACAP enhanced spatial and recognition memory deficits in R6/1 mice (Cabezas-Llobet et al., 2018). Interestingly, and according with our findings, Ladjimi M H. and collaborators found that A1 failed in improving spatial memory evaluated by the Morris Water Maze (MWM) test after an ITI of 24 hours in Wistar rats (Ladjimi et al., 2019). Altogether, these results suggest that A1 is not as powerful as PACAP and A2 inducing durable spatial memories. Remarkably, we found that PACAP analogues did not affect the locomotor activity neither induced anxiety behavior in WT nor R6/1 mice. These results are important since the activation of PACAP receptors has been found to play a role in the regulation of psychomotor behaviors (Hashimoto et al., 2001) as well as to increase anxiety-like behavior (Hammack et al., 2003; Telegdy and Adamik, 2015). Overall, we demonstrated that PACAP analogues can hippocampal-dependent cognitive function in R6/1 mice, being A2 able to preserve spatial memories for longer period of times

and that there is not an affection in locomotor activity, neither in anxiety behaviour after the treatment with analogues.

Accumulating evidence indicates that hippocampal neuroarchitecture is affected at functional and structural levels and associated with impaired learning and memory in animal models of HD (Murphy et al., 2000; Nithianantharajah et al., 2008; Milnerwood et al., 2013; Anglada-Huguet et al., 2014; Miguez et al., 2015; Giralt et al., 2017). Dendritic spine loss in the *Cornu Ammois 1* (CA1) region is the main structural plasticity change observed in the hippocampus in HD mouse models (Milnerwood et al., 2013; Miguez et al., 2015; Giralt et al., 2017). Importantly, neuroplasticity in CA1 plays an essential role in the acquisition and retrieval of spatial memory, as it has been revealed in different studies in rodents and patients (Tsien et al., 1996; Zhang et al., 2013; Stevenson et al., 2018; Dong et al., 2021). Our results demonstrated that PACAP analogues rescued dendritic spine loss of pyramidal neurons located in this CA1 region of R6/1 mice. Interestingly, PACAP has been proposed to regulate dendritic spine morphogenesis, as PACAP-deficient mice show reduced spine density and atypical morphology in the hippocampal CA1 (Atsuko Hayata-Takano et al., 2019). In addition, the capacity of PACAP to change structural plasticity has been demonstrated in hippocampal neurons, as it stimulates axon outgrowth and increases the size and density of dendritic spines (Ogata et al., 2015; Atsuko Hayata-Takano et al., 2019). Altogether, these results suggest that PACAP analogues are likely to improve spatial memory function by enhancing the structural plasticity in the hippocampus of R6/1 mice.

Dysfunction of hippocampal excitatory synapses has been described in different HD mice and associated with long term potentiation (LTP) deficits and cognitive decline (Murphy et al., 2000; Giralt et al., 2017; Quirion and Parsons, 2019; Smith-Dijak et al., 2019; Wilkie et al., 2020). Our results demonstrated that R6/1 mice had fewer excitatory synapses than WT mice in the hippocampal CA1, CA3, and dentate gyrus (DG) regions. In these animals, we observed a reduction in the number of postsynaptic

density protein 95 (PSD-95) particles without differences in synaptophysin presynaptic particles, suggesting that the postsynaptic component plays a major role in the reduction of synaptic density in R6/1 mice. These results support the idea of different authors who stand that synaptic plasticity deficits in HD mouse models are mainly due to postsynaptic dysfunction (Hodgson et al., 1999; Murphy et al., 2000). Remarkably, we found that treatment with A1 and A2 rescued the number of excitatory synapses in the CA1 and DG, respectively. Importantly, in contrast to what we previously found with PACAP, treatment with analogues does not increase the number of pre- and post-synaptic particles, but only the number of synapses. These outcomes support the idea that PACAP analogues promote different effects that differ from those of PACAP. In addition, the fact that A1 and A2 increased synaptic density in different regions of the hippocampus suggests that their action may be region-specific, which could determine their different capacities in improving spatial memory deficits.

N-methyl-D-aspartic acid receptors (NMDAR) are heteromers glutamatergic receptors consisting of GluN1, GluN2 (A-D), and GluN3 (A-B) subunits, with a key role in the regulation of LTP (Lau and Zukin, 2007; Rebola et al., 2010; Hunt and Castillo, 2012). A decrease in total and tyrosine phosphorylated GluN2A levels and a decrease in tyrosine phosphorylation of GluN2B have been described in R6/1 mice at 20 weeks (Giralt et al., 2017). Here, we studied the levels 5 weeks before, at 15 weeks of age, and our results did not show significant differences between vehicle-treated WT and R6/1 mice in the phosphorylated or total protein levels of these subunits. Remarkably, we found that A1 induce the phosphorylation of GluN2A at tyrosines 1246 and 1325, two residues related to an increase of current flux through NMDAR (Köhr and Seeburg, 1996; Taniguchi et al., 2009). In addition, treatment with A1 increased the phosphorylation of tyrosine 1472 of the GluN2B subunit. This phosphorylation allows GluN2B containing NMDAR to be placed at the postsynaptic density, facilitating the LTP (Rostas et al., 1996; Prybylowski et al., 2005; Hallett et al., 2006). In contrast, we did not observe any effect of A2 administration on total and phosphorylated levels of

GluN2A and GluN2B. Thus, our results suggest that A1, and not A2, potentiates the NMDAR function and facilitates its postsynaptic density localization in the hippocampus of R6/1 mice. Interestingly, PACAP has been described to play a role in synaptic plasticity by modulating NMDAR function. In rats, PACAP action in improving spatial memory involved GluN2B-containing NMDAR (Ladjimi et al., 2020). In accordance, in cultured hippocampal neurons PACAP induced the phosphorylation of GluN2B and enhanced NMDAR potentials (Yaka et al., 2003). Importantly, this action in CA1 pyramidal neurons has been described to be mediated by PAC1R through the PLCβ1/PKC/Pyk2/ Src signal cascade (Macdonald et al., 2005; MacDonald et al., 2007). Therefore, we hypothesized that A1 likely modulates NMDAR function in the hippocampus of R6/1 mice through the same mechanisms after the activation of PAC1R.

BDNF is known to regulate both short-term synaptic function and LTP in the hippocampus (Minichiello et al., 1999). The role of BDNF depends on its receptors Tropomyosin receptor kinase B (TrkB) and p75 neurotrophin receptor (p75NTR), by which BDNF can facilitate synaptic plasticity or activate neurodegenerative signalling, respectively (Minichiello et al., 2002; Chao, 2003; Reichardt, 2006). Several studies have reported that hippocampal levels of BDNF and TrkB receptors are reduced in different mouse models of HD (Brito et al., 2014; Miguez et al., 2015; Anglada-Huguet et al., 2016c; Cabezas-Llobet et al., 2018; Pérez-Sisqués et al., 2022), whereas p75NTR is upregulated (Brito et al., 2014). Our results showed a decrease in BDNF protein levels at 15 weeks of age. However, we did not find significant differences in the levels of TrkB and p75NTR between genotypes, suggesting that at 15 weeks of age protein levels of BDNF receptors still remain unaltered in R6/1 mice. We previously reported that PACAP intranasal administration could restore BDNF protein levels in the hippocampus of R6/1 mice (Cabezas-Llobet et al., 2018). The present study revealed that PACAP analogues do not possess this capacity. Similar results were recently obtained in Wistar rats, in which treatment with PACAP, but not A1, increased hippocampal protein levels

of BDNF (Ladjimi et al., 2019). Additionally, PACAP analogues did not cause significant changes in the protein levels of TrkB and p75NTR receptors in R6/1 mice. Altogether, our study indicates that PACAP analogues do not enhance the expression of BDNF and its receptors in the hippocampus of R6/1 mice. However, it is important to note that it has been shown that PAC1R can induce the tyrosine phosphorylation of the TrkB receptor in hippocampal neurons (Lee et al., 2002). This crosstalk between receptors involves Src kinases and allows the stimulation of BDNF signalling without altering the protein levels of BDNF and TrkB (Lee et al., 2002). Therefore, it would be particularly interesting to investigate whether A1 and A2 can induce PAC1R-mediated transactivation of TrkB, which ultimately would enhance synaptic plasticity.

Our previous results indicated that the reduction in PAC1R protein levels is a molecular alteration occurring in different models of HD and that the beneficial effect of PACAP in R6/1 mice was associated with increased PAC1R protein levels (Cabezas-Llobet et al., 2018). Here, we did not observe an increase of hippocampal PAC1R levels after the treatment with PACAP analogues. In fact, in the case of A1 treatment, we observed a tendency for PAC1R protein levels to decrease in R6/1 mice. PACAP has been described as a regulator of its own expression as well as the expression of its receptors (Shintani et al., 2005; Rat et al., 2011; Georg et al., 2016). Thus, we suggest that the reduction in PAC1R hippocampal protein levels after A1 treatment could be the result of a negative feedback stimulation of PAC1R, perhaps to avoid overstimulation, since A1 is considered a super agonist of PAC1R. Altogether these results indicate that it is not necessary to recover the protein levels of PAC1R to improve hippocampal-dependent cognitive function and neuroplasticity, suggesting that the stimulation of PACAP receptors is enough.

CREB is essential for activity-induced gene expression that mediates memory formation (Bourtchuladze et al. 1994; Silva et al. 1998). The transcriptional activity of CREB depends on the recruitment of its coactivators, such as CBP (Chrivia et al., 1993; Bourtchuladze et al., 1994; Ravnskjaer et al., 2007) and the CREB regulator

transcription co-activator 1 (CRTC1) (Parra-Damas et al., 2014). Interestingly, transcriptional deregulation of CREB-dependent specific genes involved in synaptic plasticity, together with a reduction in CBP protein levels, has been proposed to contribute to cognitive deficits in HdhQ7/Q111 mice (Giralt et al., 2012a). Accordingly, we found reduced phosphorylated CREB protein levels in CA3 and reduced CBP protein levels in the hippocampus of R6/1 mice. Interestingly, our results demonstrated that only A2 treatment induced CREB phosphorylation, specifically in the DG of R6/1 mice. Additionally, this effect was not associated with an increase in hippocampal CBP protein levels. Although we previously suggested that CBP upregulation is one of the key mechanisms by which PACAP enhances the expression of CREB-dependent genes (Solés-Tarrés et al., 2022), our results support the idea that there are multiple mechanisms by which PACAP receptors can activate CREB. Thus, A2 could favour CBP-binding to CREB, as has been described for PACAP in activated microglia (Delgado, 2002b), or induce nuclear translocation of the co-activator CRTC1, as has been observed in cortical neurons treated with PACAP (Baxter et al., 2011). On the other hand, A2 could also be acting through different protein kinases associated with PACAP receptors such as PKA, Ca^{2+}/calmodulin-dependent protein kinase (CaMK), and mitogen-activated protein kinase (MAPK), that have been reported to directly phosphorylate CREB (Alberini, 2009). Overall, our study suggests that A2 could promote CREB-mediated transcriptional activity in DG by inducing CREB phosphorylation specifically in this hippocampal region. Interestingly, previous studies have demonstrated that PACAP-PAC1 signalling can cause long-lasting changes in neuronal excitability, specifically in DG granule cells (Johnson et al. 2020a). In addition, PACAP-mediated activation of the DG appears to enhance the retention of contextual memory (Johnson et al., 2020a, 2020b). Thus, it is tempting to suggest that this activation of CREB in the DG, mediated by A2 and not A1, could contribute to the improvement of a more long-term memory observed in A2-treated mice.

The presence of mHTT aggregates is the histopathological hallmark of HD (Arrasate and Finkbeiner, 2012). Aggregation of mHTT occurs in the hippocampus of R6/1 mice, and the level of mHTT aggregates in CA1, CA3, and DG correlates with phenotypic severity (Cabanas et al., 2020). Importantly, we found that R6/1 mice treated with PACAP analogues showed reduced number of mHTT aggregates in the DG. In addition, in A1-treated R6/1 animals, we observed a reduction in mHTT aggregates in the CA1 and CA3 hippocampal regions. Thus, our results suggest that the reduction in mHTT aggregates, especially in the DG might be determining for cognitive improvement. Accordingly, it has been shown that focal expression of mHTT in the DG is sufficient to lead to the appearance of mHTT aggregates, long-term spatial memory impairments, and changes in hippocampal activity (Schwab et al., 2017). Interestingly, our results agree with those of our previous study, in which we found that PACAP-induced improvements in cognitive function in R6/1 mice were associated with a reduction in the number of mHTT hippocampal aggregates (Cabezas-Llobet et al., 2018). Additionally, it has been found that different compounds that suppress mHTT aggregates are neuroprotective in HD (Ferrante et al., 2002; Chen et al., 2011; Anglada-Huguet et al., 2014; Barriga et al., 2017). Remarkably, the fact that we did not detect changes in the volume of aggregates suggests that PACAP analogues inhibit the aggregation process instead of increasing the mHTT clearance. Therefore, we can conclude that PACAP analogues might prevent cognitive decline by reducing mHTT aggregation in the hippocampus of R6/1 mice.

In summary, we provide evidence for the first time that intranasal administration of PACAP analogues improves cognitive function in HD. Specifically, we described that although both analogues improve spatial memory deficits in R6/1 mice, the effects of A2 appear to be more long-lasting. Importantly, both analogues enhance hippocampal structural plasticity studied in the CA1 region. In addition, PACAP analogues also increase the excitatory synaptic density but in different areas of hippocampus. While this effect of A1 seems to be restricted to CA1, A2 acts mainly in the DG. A different

effect of A1 and A2 was also observed in the mechanism of action. The beneficial effect of A1 was associated to an increase of postsynaptic density localization of NMDAR, potentiating their function, and the beneficial role of A2 was related to the activation of CREB, particularly in DG. Importantly, A1 and A2 reduce the number of mHTT aggregates in the hippocampus. Overall, our results clearly show therapeutic capacity of both analogues in improving cognitive phenotype and hippocampal function in HD. Interestingly, A1 and A2 initiate different molecular events involved in neuroplasticity, and their actions seem to be region-specific (Figure 37).

PACAP analogues also have the capacity to improve motor function in R6/1 mice.

HD is usually diagnosed when movement difficulties occur. Different motor disturbances appear in HD mouse models (Carter et al., 1999; Crook and Housman, 2011). Here, we studied the effects of PACAP analogues in R6/1 mice at 19 weeks of age, when motor impairments are already present (Van Dellen et al., 2008; Brooks et al., 2012c; Cabanas et al., 2020). We first observed that PACAP analogues significantly improved the balance in R6/1, as evaluated by the balance beam test. In addition, treated R6/1 mice performed better in the rotarod task, which validates that treatment with PACAP analogues also improve motor coordination. These findings agree with the accumulating evidence that PACAP receptors activation can prevent motor and behavioral deficits in different murine models of neurodegenerative disorders including HD (Tamás et al., 2006; Solés-Tarrés et al., 2022) and PD (Reglodi et al., 2004a; Shivers et al., 2014).

Motor impairments in patients undergoing HD have been correlated with striatal atrophy and the loss of the medium spiny-like neurons (MSNs) (Vonsattel et al., 1985; Raymond et al., 2011; Guo et al., 2012) Similarly, motor disturbances occurring in HD mouse models are associated with progressive disconnection between the cortex and striatum (Bunner and Rebec, 2016; Fernández-García et al., 2020), as well as striatal volume loss associated to the MSNs death or atrophy, depending on the mouse model

(Slow et al., 2003; Saxena et al., 2020). Our results showed that motor disturbances in R6/1 mice were accompanied by a significant decrease in striatal volume, reduced number and size of dopamine- and cAMP-regulated phosphoprotein 32 kDa (DARPP-32) positive MSNs cells in the striatum, and reduced intensity of DARPP-32, as it has been described before (Rattray et al., 2013; Anglada-Huguet et al., 2014; Barriga et al., 2017). While some authors have reported that neuronal death does not occur in the brains of R6/1 mice (Hansson et al., 1999; Crook and Housman, 2011), other studies support the idea that neuronal loss is happening in the striatum of R6/1 mice (Bayram-Weston et al., 2012; Rattray et al., 2013). The reduction in the number of DARP-32 positive cells we observed in R6/1 mice suggests that neuronal loss could be happening in their striatum, but it could also be due to the reduced intensity of DARPP-32. Importantly, although PACAP analogues could not rescue striatal volume loss in R6/1 mice, we observed that A1 partially rescued the number of DARPP-32 positive MSNs cells and prevented neuronal atrophy. Since the partial rescue of DARPP-32 positive cells was not accompanied by the increase of DARPP-32 intensity, our outcomes suggest that A1 may prevent from neuronal loss. Altogether, we can conclude that A1 but not A2 exerts protective effects on the MSNs population. This agrees with previous studies demonstrating that PACAP receptors activation offers neuroprotection to striatal neurons (Tamás et al., 2006b; Solés-Tarrés et al., 2020) and increases neuronal soma size in PC12 cells (Ravni et al., 2008). Importantly, different authors have demonstrated that PACAP can promote other morphological changes, such as neuritogenesis, in SH-SY5Y (Monaghan et al., 2008), PC12 (Sakai et al., 2001), cerebellar granule (Gonzalez et al., 1997), and dorsal root ganglionic cells (Nielsen et al., 2004). In addition, in this work we have described that PACAP analogues prevent dendritic spine loss in the hippocampus. Therefore, it would be particularly interesting to investigate the effects of A1 and A2 on the dendrite pathology that occurs in MSNs of R6/1 mice (Spires et al., 2004a).

Deficits in neurotrophic support have been proposed to underlie the vulnerability of MSNs to degeneration in HD. Accordingly, deficits in striatal BDNF transcription and protein levels have been well documented in human HD patients and in different mouse models of HD (Zuccato et al., 2001, 2005; Luthi-Carter et al., 2002; Gines et al., 2003b; Hermel et al., 2004; Spires et al., 2004b; Apostol et al., 2008; Gharami et al., 2008; Giralt et al., 2011a; Simmons et al., 2011). Our results did not show reduced striatal BDNF protein levels in the R6/1 mice and we did not observe increased BDNF expression after treatment with PACAP analogues. Importantly, we previously found that treatment with PACAP restores striatal BDNF protein levels in R6/1 mice (Solés-Tarrés et al., 2022). The fact that PACAP analogues could not perform this action agrees with what we found in the hippocampus, supporting the idea that their biomolecular action differs from that of PACAP and does not involve BDNF upregulation. Interestingly, similar results were obtained by Ladjimi and colleagues previously, when they showed that PACAP, but not A1, increased BDNF protein levels in Wistar rats (Ladjimi et al., 2019, 2020). It has not escaped our attention that while our previous results in STHdh cells indicated that PAC1R activation is necessary for BDNF upregulation, these two analogues with increased affinity for PAC1R do not perform this action. This result suggests that VPACR activation may be as necessary as PAC1R activation in increasing BDNF levels. However, it is worth considering that, together, our data demonstrate that behavioural improvement mediated by the activation of PACAP receptors can occur in HD without the recovery of BDNF protein levels. This is especially interesting since it reinforces the idea that PACAP receptors, by themselves, can activate a wide diversity of neuroprotective mechanisms in HD.

Accumulating evidence indicates that deficits in neurotrophic support occurring in HD are not only due to low BDNF protein levels but also due to the impairment of BDNF downstream signaling, which is initiated by TrkB and p75NTR receptors. An imbalance between TrkB and p75NTR has been described in the striatum of different HD mouse models as well as in the putamen of HD patients (Brito et al., 2013; Simmons et al.,

2016). Our results did not show significant differences in TrkB and p75NTR protein levels in the striatum of R6/1 mice treated with or without PACAP analogues at 20 weeks of age. However, it is important to mention that normal levels of TrkB and p75NTR, as well as unchanged levels after treatment with PACAP analogues, do not necessarily reflect the normal or unchanged function of these receptors. Therefore, we studied the activation of the three principal pathways associated with the TrkB receptor: Akt, ERK1/2, and PLCγ1 (Chao, 2003; Huang and Reichardt, 2003; Sasi et al., 2017). Importantly, we found that treatment with A1 specifically increased the activation of Akt in the striatum of R6/1 mice, as we did not observe any changes in the activation of ERK1/2 and PLCγ1. The activation of Akt is one of the central mechanisms by which neurons promote its survival by phosphorylating several substrates (Manning and Cantley, 2007). Interestingly, it has been suggested that activation of the PI3K/Akt pathway may ameliorate the detrimental effects of mHTT expression (Saavedra et al., 2010; Creus-Muncunill et al., 2018). In addition, Akt activation has been related to the amelioration of motor performance in different mouse models of HD (Simmons et al., 2013; Lopes et al., 2014). Therefore, it is tentative to suggest that the activation of Akt underlies the neuroprotective actions of A1 in MSNs. The fact that we did not observe a neuroprotective action of A2 in MSNs or increased phosphorylated levels of Akt supports this hypothesis. Interestingly, we previously observed that Akt mediates, at least in part, the PACAP-induced anti-apoptotic effect in Q111 cells, an action that has also been described in cerebellar granule neurons (Bhave and Hoffman, 2004). In additiony, it has been demonstrated that PACAP increases activated Akt through PAC1R-mediated induction of the tyrosine activity of TrkB under neurotrophic deprivation (Lee et al., 2002). Therefore, this could be a possible mechanism by which A1 activates the striatal Akt pathway in R6/1 mice.

PAC1R is the specific PACAP receptor, and by which PACAP has been suggested to promote beneficial effects. We previously observed that PAC1R is downregulated in the striatum of R6/1 mice as well as in Q111 cells (Solés-Tarrés et al., 2022). Here, we

found that beneficial effect promoted by A1 in the striatum was associated with the partial recovery of PAC1R protein levels. At this point, we can note that our results regarding PAC1R protein levels after treatment with PACAP or PACAP analogues are diverse. The beneficial effects of PACAP in R6/1 mice have been shown to be related to an increase in PAC1R in both the hippocampus and the striatum. However, in Q111 cells, the PACAP-induced beneficial effects were not accompanied by this increase. Related to analogues, A2 did not affect PAC1R protein levels in any brain area. In contrast, we observed a tendency for PAC1R protein levels to decrease in the hippocampus of A1-treated R6/1 mice, whereas in the striatum of A1-treated R6/1 mice we observed a partial recovery. Overall, our findings support the idea that A1 may exert regulatory actions on the expression of PAC1R, as described for PACAP (Shintani et al., 2005; Rat et al., 2011; Georg et al., 2016). However, our results highlight the need to investigate the activity of PAC1R after treatment with PACAP and PACAP analogues to better understand the role of PAC1R in the biological effects induced by peptides.

Evidence has shown that the activity of CREB and CBP proteins in the striatum is decreased in HD, which determines its vulnerability (Steffan et al., 2000; Nucifora et al., 2001; Choi et al., 2009). In fact, manipulation of the CREB transcriptional pathway has been suggested as a promising approach for the amelioration of HD (Choi et al., 2009). Importantly, PACAP receptors are linked to different pathways that can lead to transcriptional modifications through CREB activation (Vaudry et al., 2009). In addition, we previously found that PACAP-promoted beneficial effects were associated with increased CBP protein levels in the striatum of R6/1 mice and Q111 cells (Solés-Tarrés et al., 2022). Therefore, we investigated whether PACAP analogues could also promote this effect. Our results demonstrated that PACAP analogues did not significantly change striatal CBP protein levels. Again, our results agree with the effect of PACAP analogues in the hippocampus, supporting the idea that PACAP analogues differ from PACAP in their molecular action. However, this result does not necessarily reflect the

inability of PACAP analogues to induce transcriptional changes via CREB. Therefore, it would be interesting to evaluate striatal protein levels of phosphorylated CREB as well as the expression of CREB-dependent genes.

The aggregation of mHTT occurs in the striatum of R6/1 mice at around 8 weeks of age, and the proportion of cells displaying inclusions increases gradually with age (Hansson et al., 2001; Li et al., 2005). Interestingly, we found that A1, but not A2 administration, significantly reduced the number of aggregates in the striatum of R6/1 mice. In addition, since we did not find any changes in the volume of mHTT aggregates, our results suggest that A1 inhibits mHTT aggregation. Importantly, this is the first study to demonstrate that activation of PACAP receptors can result in a reduction in the number of mHTT aggregates in the striatum. Although the role of mHTT aggregates in the brain remains unclear, different studies have demonstrated that drugs targeting mHTT aggregates/mHTT aggregate formation are promising. For example, Congo red can reduce the aggregation of mHTT and has been demonstrated to improve motor function, reduce weight loss, and increase the lifespan of R6/2 mice (Yamamoto et al., 2000; Sánchez et al., 2003). On the other hand, a benzothiazole derivative named riluzole inhibited the number and size of mHTT aggregates in the striatum of R6/2 mice, which was associated with a 17% delay in weight loss and a 10% increase in survival time (Heiser et al., 2002; Schiefer et al., 2002). Importantly, it has been demonstrated that the activation of Akt inhibits the formation of mHTT intranuclear inclusions by directly phosphorylating HTT at serine 421 (Humbert et al., 2002). Therefore, it is tentative to suggest that a possible mechanism by which A1 protects MSNs is inhibiting mHTT aggregation after Akt activation. The fact that A2 cannot protect MSNS, activate Akt, or inhibit mHTT aggregation supports this hypothesis.

In summary, we have described that intranasal administration of PACAP analogues improves motor function in the R6/1 mouse model of HD. Importantly, A1 protects MSNs cells, induces the activation of the pro-survival pathway Akt, and reduce the number of mHTT aggregates in the striatum of R6/1 mice. Unfortunately, we have not

been able to decipher the mechanisms by which A2 promotes improvement of motor function (Figure 37). It is important to note that intranasal administration does not allow the reach of a specific brain area. In addition, PAC1R expression in the mice neocortex is extensive (Zhang et al., 2021), and we previously found that PAC1R protein levels were reduced in the motor cortex of R6/1 mice (Solés-Tarrés et al., 2020). Therefore, we suggest that A2 may promote beneficial effects in other areas affected by HD resulting in the recovery of motor phenotype we have observed.

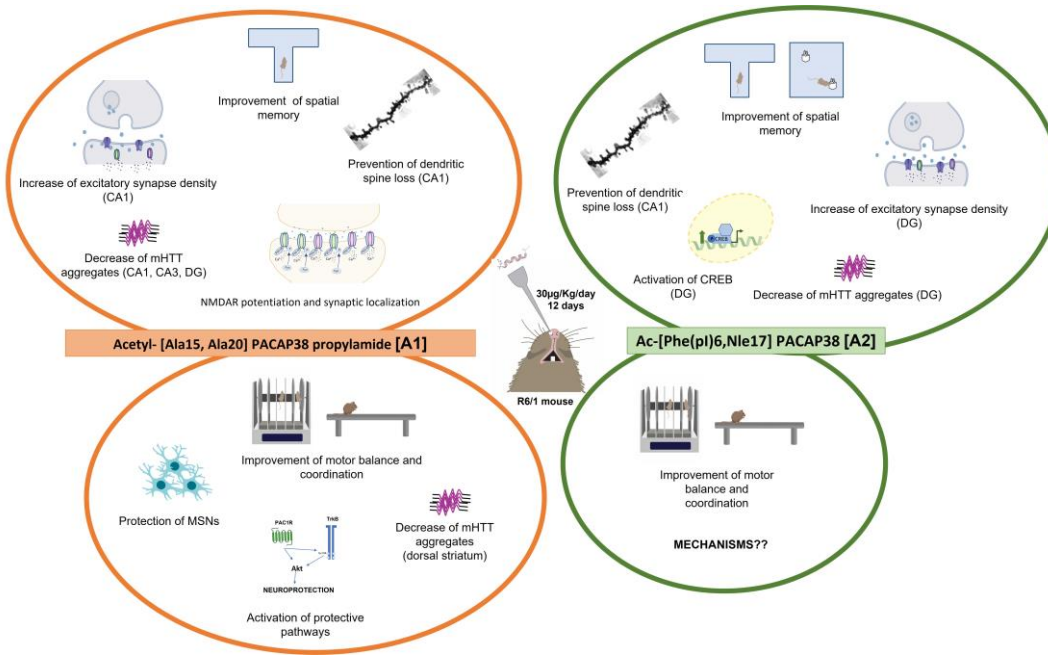


Figure 37: Therapeutic capacities of PACAP analogues in the symptomatology and neuropathology of HD. Schematic representation of the beneficial actions of PACAP analogues after intranasal administration for 12 days in an R6/1 mouse model of HD. A1 (orange) improved spatial learning and memory deficits evaluated by the T-MAZE test enhancing structural plasticity and increasing synaptic density in the CA1 region. A1 was likely to improve hippocampal synaptic plasticity by promoting postsynaptic density localization of N-methyl-D-aspartic acid receptors (NMDAR) and potentiating its function. Moreover, A1 reduced the number of mutant huntingtin (mHTT) aggregates in all hippocampal regions studied. Importantly, A1 was also effective in improving motor deficits and protecting medium spiny-like neurons (MSNs) cells by inducing Akt activation and inhibiting mHTT aggregation in the dorsal striatum of R6/1 mice. Alternatively, A2 (green) improved spatial memory deficits evaluated the T-MAZE test and the novel object location test (NOLT) by enhancing the structural plasticity in CA1. Interestingly, A2 had its main effect in the DG, where it increased synaptic density, induced cAMP-response element-binding protein (CREB) activation, and reduced mHTT aggregation. Additionally, A2 improved motor disturbances in R6/1 mice through undeciphered mechanisms.

General discussion

The description of therapeutic targets is a challenge in the field of Huntington's disease, as no curative treatment is available. In the present study, we described PAC1R as a potential therapeutic target in the pathology of HD based on two findings. Firstly, we found reduced protein levels of PAC1R in Q111 cells as well as in the striatum of R6/1 mice. Secondly, we determined that after PACAP treatment, PAC1R signaling enhances the activation and expression of neuroprotective and neurotrophic proteins in Q111 cells.

Since the pharmacological use of PACAP is limited owing to its rapid degradation and lack of selectivity for its receptors, metabolically stable analogues with improved affinity for PAC1R appear attractive compounds to ascertain its usefulness in clinical conditions. Importantly, the findings of our study clearly show the therapeutic potential of two novel stable PACAP analogues with high affinity and potency for PAC1R. For the first time, we found that intranasal administration of PACAP analogues improved cognitive and motor function in R6/1 mice. These improvements were associated with structural and molecular changes in the hippocampus and striatum; however, we found that A1 and A2 differ in their mechanisms of action. We believe that this mechanistic difference between analogues can be explained by several reasons. First, modifications to the PACAP molecule may affect its distribution in the brain. Previous studies have demonstrated that administered intranasally, PACAP is transported across the blood- brain barrier (BBB) and distributed in the whole brain, including striatum and hippocampus (Nonaka et al., 2012). However, we did not observed changes in the striatum after A2 treatment, indicating it may have a different distribution pattern of PACAP and A1. In addition, we observed a region-specific action of PACAP analogues in the hippocampus. On the other hand, both analogues have higher affinity for PAC1R, however, they may have different potencies in activating the associated pathways, leading to different molecular consequences. Lastly, it is also important to consider that although PACAP analogues have increased affinity for

PAC1R, they preserve the capacity to bind to VPACR. Therefore, VPACR activation likely contributes differently to the beneficial action of PACAP analogues. This idea is supported by the results from our first study, in which we demonstrated the advantageous effects of VPACR activation in Q111 cells. Importantly, these reasons explaining the differences between A1 and A2 may also explain why their actions also diverge from what we previously described for PACAP.

Finally, our study reinforces the idea that intranasal administration is a suitable method for delivering PACAP derivatives to the brain, which agrees with the results of previous studies on PACAP (Rat et al., 2011; Nonaka et al., 2012; Cabezas-Llobet et al., 2018; Solés-Tarrés et al., 2022). Importantly, since this form of administration is non-invasive, easy-to-use, and allows the drug to reach the brain rapidly with minimal side effects, it is widely accepted in human studies (Reglodi et al., 2018). In addition, intranasal delivery is currently considered a potential approach for treating chronic neurodegenerative diseases, such as AD (Gomez et al., 2012).

Limitations of the study

It is necessary to point out some limitations that have not been possible to control because of the intrinsic nature of the investigation or solve because of the available amount of time and resources.

As in most biomedical investigations, the choice of model used is a critical step. In our *in vitro* studies, the STHdh cellular model allowed us to determine the role of PACAP receptors against the toxic insult of mHTT. Although widely accepted, these cells do not fully replicate the properties of neuronal cells *in vivo*. In addition, it is important to consider that PACAP receptors are expressed not only in neurons but also in glial cells. Therefore, our study using the STHdh cells does not fully represent how all PACAP receptors participate in neuroprotection *in vivo*.

In our *in vivo* studies, we only used male mice, which is an important limitation because females might respond differently to treatment. In fact, it has been demonstrated that there are sex differences in the expression of PACAP and PAC1R in the brain, which could mean that males and females might require different dosages of PACAP analogues. Overall, our study presents a male bias and may not be applicable to women.

Finally, the fact that we restricted our research to the hippocampus and striatum suppose another limitation. Since PACAP receptors are expressed through the brain and intranasal administration does not allow specific delivery to the striatum and hippocampus, the action of PACAP analogues in other brain areas is highly likely to participate in the behavioral performance of R6/1 mice. Therefore, it would be interesting to further understand the regional and biological actions of these analogues in future studies. A method to determine the distribution pattern and durability of PACAP analogues in the brain consists of intranasal administration of A1 and A2 labelled radioactively with ^{131}I and quantifying the level of radioactivity in different regions of interest at different time points. This study is particularly interesting because it could help us understand the pharmacodynamics and pharmacokinetics of PACAP analogues after its intranasal administration, which has never been studied.

CHAPTER VI: CONCLUSIONS

The results obtained from the current thesis led us to conclude that:

1. PACAP protects striatal cells from mHTT-mediated toxicity.
2. Although all PACAP receptors may participate in PACAP-promoted antiapoptotic action, the specific activation of PAC1R plays a key role promoting the activation of the pro-survival pathways ERK1/2 and Akt, and the expression of neuroprotective and neurotrophic proteins in striatal cells.
3. Intranasal administration of PACAP analogues improves cognitive and motor function in the transgenic R6/1 mouse model of HD.
4. In the hippocampus, the two PACAP analogues enhance neuroplasticity having a regional action and through different mechanisms. Both analogues increase the number of dendritic spines and excitatory synapses. However, while A1 promotes the synaptic localization of NMDAR and enhances their function, A2 induces the phosphorylation of CREB in the DG.
5. In the striatum, A1 but not A2, protects medium spiny neurons. This effect is associated to the increase of PAC1R and Akt phosphorylation.
6. The two PACAP analogues reduce the number of mHTT aggregates in the hippocampus, while only A1 decrease the number of mHTT aggregates in the striatum.

The general conclusion of this thesis is that PAC1R is a potential therapeutic target for HD and that the intranasal administration of metabolically stable analogues of PACAP displaying higher affinity for PAC1R represents a promising pharmacological strategy to fight cognitive and motor symptoms of HD.

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