



INFLUENCE OF RETINOID X RECEPTORS ACTIVATION ON THE EXPRESSION OF IONOTROPIC GLUTAMATE RECEPTORS

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder first described in 1906. The disease is characterized by the progressive and irreversible loss of neurons in the central nervous system (CNS) leading to loss of cognitive functions, including memory. The current paradigm of AD includes two main lesions, being the intracellular neurofibrillary tangles and the extracellular amyloid β ($A\beta$) plaques. Numerous therapies aimed at reducing the amyloid deposits through various treatments were conducted. It was shown that while the cellular symptoms were indeed impacted, the cognitive functions of the patients were not well reestablished.

In 2015, professor Octave with N. Pierrot (phD) and their team shed light on the case of a patient suffering from AD and was given a treatment commonly used against cutaneous T cell lymphoma, with the consequences of improved cognitive functions. This treatment was bexarotene, an agonist of retinoid X receptors acting on lipid homeostasis. The purpose of this work was to try to understand the underlying mechanisms behind the beneficial effects of bexarotene on cognitive functions. This was performed through the analysis of the expression of various genes proven to be essential in the establishment of memory processes, including genes coding for subunits of receptors involved in synaptic plasticity (strengthening of neuronal junctions). In addition, the expression of a cholesterol transporter was also assayed as its role in bexarotene action has been proven.

Primary cultures of hippocampal neurons of rat embryos were treated with bexarotene for 24 hours before harvesting, allowing the comparison of relative gene expression against non-treated neurons (control group). Reverse transcription and quantitative polymerase chain reaction were carried out to measure the messenger RNA and western blotting was used to measure the relative expression of proteins.

The results gathered throughout this study suggest the modification of subunit ratio of NMDA (N-methyl-D-aspartate) synaptic receptors as a potential part of bexarotene mode of action. Some effects on AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors were also observed but no consistent explanation could be drawn.

Index

ACKNOWLEDGEMENTS.....	2
ABSTRACT.....	3
I) <u>INTRODUCTION</u>	6
1) <i>Alzheimer's disease</i>	6
General.....	6
Neurofibrillary tangles.....	6
Senile plaques and amyloid peptide.....	8
Presenilins.....	10
Apolipoprotein E.....	10
2) <i>Hippocampus</i>	12
3) <i>Neurotransmissions</i>	13
Excitatory and inhibitory neurotransmissions.....	13
Synaptic plasticity.....	15
AMPA Receptors.....	16
NMDA Receptors.....	17
4) <i>Nuclear receptors</i>	19
RXR.....	19
LXR.....	20
PPAR.....	21
II) <u>OBJECTIVES</u>	22
III) <u>MATERIAL ET METHODS</u>	23
1) <i>Primary Culture of hippocampal neurons</i>	23
1.1. Principle.....	23
1.2. Protocol.....	23
2) <i>RNA extraction</i>	24
2.1. Principle.....	24
2.2. Protocol.....	24
3) <i>Retrotranscription</i>	25
3.1. Principle.....	25
3.2. Protocol.....	25
4) <i>Quantitative polymerase chain reaction (qPCR)</i>	26
4.1. Principle.....	26
4.2. Protocol.....	28
5) <i>Western blotting</i>	28
5.1. Protein assay.....	29
5.2. Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE).....	29
5.3. Nitrocellulose membrane transfer.....	30
5.4. Total protein staining.....	32
5.5. Immunostaining.....	32
<i>Statistical Analysis</i>	34
Shapiro-Wilk's w-test.....	34
Student's t-test.....	36
Mann-Whitney's u-test.....	37
IV) <u>RESULTS</u>	39
1) ABCA1.....	39
2) Ionotropic glutamate receptors.....	42
3) Synaptophysin.....	52
4) PSD95.....	54
V) <u>DISCUSSION</u>	56
1) <i>Discussion of the model</i>	56
2) <i>Discussion of the results</i>	56
2.1) ABCA1 expression increases in presence of bexarotene.....	57
2.2) The response of AMPA receptors subunits GluA1 and GluA2 to bexarotene is not well defined.....	57
2.3) Bexarotene could impact the subunit ratio of NMDA receptors.....	58
2.4) PSD 95 and Synaptophysin.....	59

VI)	<u>CONCLUSION AND PERSPECTIVES</u>	60
VII)	<u>REFERENCES</u>	61

I) Introduction

1) Alzheimer's disease

General

Alzheimer's disease (AD) is a neurodegenerative brain disease that was first presented to the 37th meeting of psychiatrists in Germany by the clinical psychiatrist and neuroanatomist Alois Alzheimer in 1906, based on observations made upon a 50 years old woman presenting signs of paranoia among other disturbances, and whose post-mortem brain analysis revealed the presence of senile plaques and neurofibrillary tangles. (Hippius and Neundörfer, 2003)

The disease is characterized by a progressive loss of central nervous system (CNS) neurons leading to a brain cortex atrophy. The loss of cognitive functions appear as the first symptoms encountered when AD patients are observed. As widely and sadly known, memory processing alterations and irreversible loss are commonly observed. On a cellular scale, some of these processes are taking place in the hippocampus region of the brain, in the limbic system. The hippocampal region (including the hippocampus and adjacent, similar structures) are thought to be one of the main site for memory processing.

Nowadays, AD is the leading cause of dementia. For example, in the United-States, one in 10 people age 65+ is suffering from AD related dementia. (Hebert and al, 2010)

The autopsies carried out on AD patients revealed two main specific lesions, the intracellular neurofibrillary tangles and the extracellular senile plaques, also referred to as neuritic plaques. Both lesions are detailed here below.

Neurofibrillary tangles

The neurofibrillary tangles refer to intracellular lesions. In physiological conditions, the neuronal axons' tubulin interacts with a protein called Tau (tubulin-associated unit). This protein, mostly present in neurons of the central nervous system, has the role of stabilizing the microtubules as well as playing a part in their flexibility (see Fig. 1 from Brunden et al., 2009). Tau protein modulation occurs partly via phosphorylation but, when this process goes beyond regular boundaries, the protein becomes hyperphosphorylated resulting in the

appearance of paired-helical filament (PHF) state of Tau. This leads to a loss of interaction between Tau and the neurons' tubulin (with the effect of microtubules dissociation) and, in turn, to cellular death. (Brion et al., 1985 ; Delacourte and Defossez, 1986 ; Grundke-Iqbal et al., 1986 ; Mudher et al., 2002)

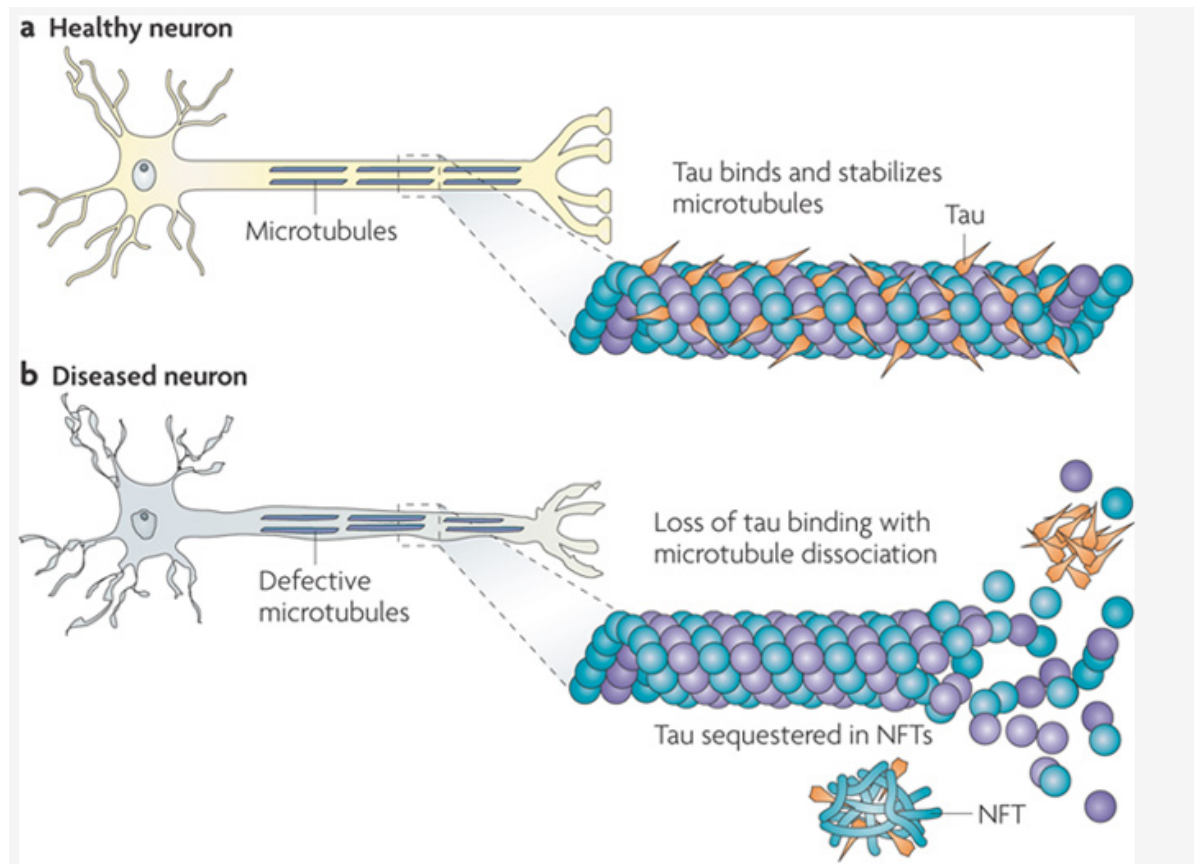


Figure 1: Effects of hyperphosphorylated Tau on microtubules and neurofibrillary tangles (NFT) formation

a) In a healthy neuron the microtubules are stabilized by the Tau protein

b) In a diseased neuron the defective Tau protein does not bind to the microtubules, leading to their dissociation as well as the sequestration of the protein in the NFTs. These defective microtubules can lead in turn to cellular death.

From Brunden et al., 2009

Senile plaques and amyloid peptide

A well known phenomenon occurring in AD patients is the presence of extracellular lesions (as opposed to the neurofibrillary tangles) called senile plaque, also referred to as amyloid plaques. These plaques consist of an accumulation of the amyloid peptide called A β resulting from the proteolytic cleavage of its transmembrane precursor APP (Amyloid Peptide Precursor). This cleavage can occur in two distinct pathways : non-amyloidogenic and amyloidogenic (Fig. 2).

In the former, APP is first cleaved by the α -secretase, releasing the soluble APP in its α form (sAPP α) and the intracellular domain along with P3 peptide (C83 fragment). This cleavage takes place in the sequence of the amyloid β . Thus releasing, during the next cleavage carried out by the γ -secretase enzymatic complex, the amyloid intracellular domain (AICD) and the P3 peptide

On the other hand, in the amyloidogenic process, the precursor is first cleaved by a β -secretase (releasing the β form of the soluble APP and the A β -containing C99 fragment) then by the γ -secretase leading this time to the formation of soluble A β that can then undergo oligomerization. In the amyloid cascade hypothesis, this increase in A β will lead to loss of function and cellular death. Furthermore, similarly to the non-amyloidogenic process, the second cleavage releases the AICD, which participates in some genes' expression regulation.

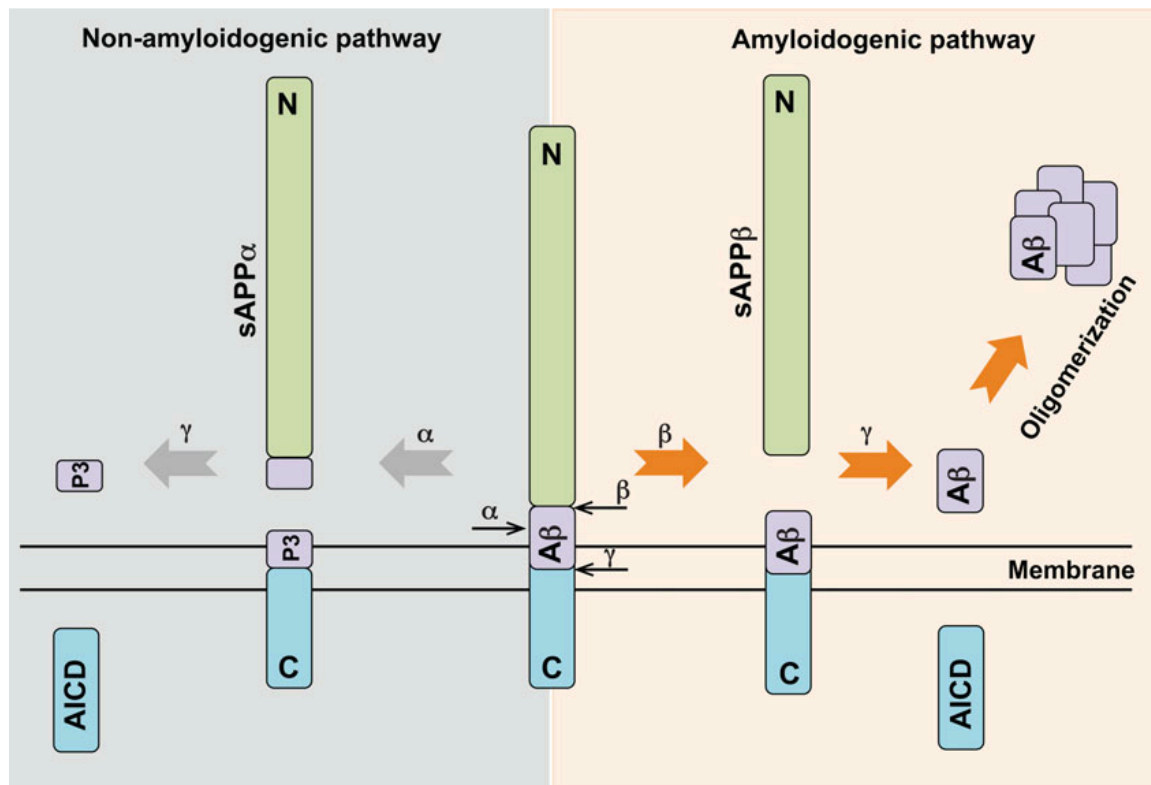


Figure 2: APP processing

In the non-amyloidogenic pathway, alpha-secretase cleaves the amyloid precursor protein, releasing the sAPP α containing the amino end (N) of APP. Then, the gamma-secretase complex cleaves the transmembrane domain releasing the P3 peptide from the amyloid intracellular domain (AICD) containing the carboxy end (C) of APP.

In the amyloidogenic pathway, beta-secretase cleaves APP, releasing sAPP β , which is itself cleaved by the gamma-secretase complex releasing A β from the AICD. This A β undergoes oligomerization and will be partly responsible for cellular death, according to the amyloid cascade hypothesis.

(Amyloid precursor protein and neural development, Maya Nicolas^{1,2,3} and Bassem A. Hassan^{1,2,3,*})

The γ -secretase complex is thus present in both pathways but its role in the amyloidogenic has been proven rather determinant. The γ cleavage generates either a 40 amino acids residue (A β ₄₀) or 42 amino acids residue (A β ₄₂), depending on between which amino acids the cleavage takes place, with the latter being more hydrophobic and susceptible to aggregate, thus more accountable for the formation of the amyloid plaques.

Among the different units of the γ -secretase enzymatic complex are the presenilins, described as the catalytic core.

Presenilins

Presenilins are a family of transmembrane proteins. Humans, and vertebrates in general, have two homologs of presenilin, PS1 encoded by PSEN1 (chromosome 14) and PS2 encoded by PSEN2 (chromosome 1) in humans. Presenilins were the first subunits described in γ -secretase both *in vitro* and *in vivo*. Their key role in the processing of APP has been shown via knock-out experiments of PS1 and PS2 in mice (De Strooper et al., 1998 ; Zhang et al., 2000)

When both genes are knocked out, there is no detectable γ -secretase activity.

Genetic studies have shown that mutations of presenilins 1 and 2, as well as APP are responsible for the major part of familial AD. On the other hand, a lipid transporter-coding gene has the particularity, depending on the expressed allele, to confer susceptibility to AD. This transporter is the apolipoprotein E (ApoE).

Apolipoprotein E

On the human chromosome 19 is the APOE gene, coding for the lipid transporter apolipoprotein E which is mainly synthesized in the liver but is also found in the brain where it is synthesized by astrocytes, where it is said to be the main cholesterol carrier, transporting cholesterol to neurons. ApoE contains 299 amino acids and possesses three main different alleles, making it polymorphic.

These polymorphisms are called $\epsilon 2$, $\epsilon 3$ and $\epsilon 4$. The differences take place as substitution of amino acids 112 and 158 which are, in $\epsilon 3$, cysteine and arginine respectively. For $\epsilon 2$ the arginine is replaced by a cysteine while in $\epsilon 4$ the cysteine is replaced by an arginine. These substitutions influence the structure of ApoE and affects its ability to bind lipids and receptors (Liu et al., 2013).

The $\epsilon 2$ form has a frequency of 8,4% and has been linked to lipid abnormalities due to its inability to properly bind to its receptors, preventing clearance of triglycerides and cholesterol from the bloodstream and leading to increased blood levels of these fats.

The $\epsilon 3$ form has a frequency of 77,9% and is considered the neutral allele of ApoE.

The $\epsilon 4$ variant has a frequency of approximately 13,7% and has been linked to different conditions such as atherosclerosis, loss of cognitive function and AD. (Liu et al., 2013)

ApoE plays a role in the clearance of $A\beta$ with the help of the ATP-binding cassette A1 (ABCA1), which is also a cholesterol transporter. ApoE is synthesized in astrocytes and microglia, and is then lipidated by ABCA1. In turn, this lipoprotein particle binds to soluble $A\beta$ and facilitates its uptake via cell receptors recognition. An increase in ApoE and ABCA1 could be an effective way to promote $A\beta$ clearance. The stimulation of certain nuclear receptors (detailed further) has been shown to increase ApoE production as well as up regulating ABCA1 (Chen et al., 2011 ; Zhao et al., 2014).

Genetics and APP

It has been demonstrated that mutations of APP, presenilin 1 and 2 genes (PS1 and PS2) are present in the genome of patients suffering from the inherited form of AD, being a small fraction of the total AD suffering population (around 1%) (Tanzi and Bertram, 2005) while the remaining 99% of AD cases are represented by the sporadic form of the disease. Accordingly, multiple studies on AD focused on APP and the $A\beta$ peptide to try to find the mechanisms underlying the development of the disease, if not discover a potential cure. In this hypothesis, different strategies were applied to try to demonstrate the $A\beta$ -linked toxicity role in AD development.

Despite being one of the leading theories as of today, it has to be stated that AD cognitive symptoms develop prior to the appearance of the senile plaques. In normal, physiological conditions, neuronal networks function synchronically while in AD patients, a loss in synchronicity has been observed. (Jeong, 2004). This neuronal dysfunction linked to atypical activity can be observed prior to the development of the documented neuropathological lesions and could therefore have a role in the decline of the cognitive processes. (Martin et al., 1994 ; Berendse et al., 2000)

2) Hippocampus

The hippocampus is a structure present in the brain of vertebrates and plays an important role in different memory mechanisms (short-term, long-term and spatial memory) (Martin, JH, 2003). Humans as well as other mammals (e.g. rodents) have two hippocampi, one in each brain hemisphere (Fig. 3 from Soya et al., 2011). The hippocampus, along with other structures such as hypothalamus, olfactory bulbs and amygdala, belongs to the limbic system, which is involved in motivation, emotion, learning and, as stated before in the case of the hippocampus, memory. (Eichenbaum, 2007)

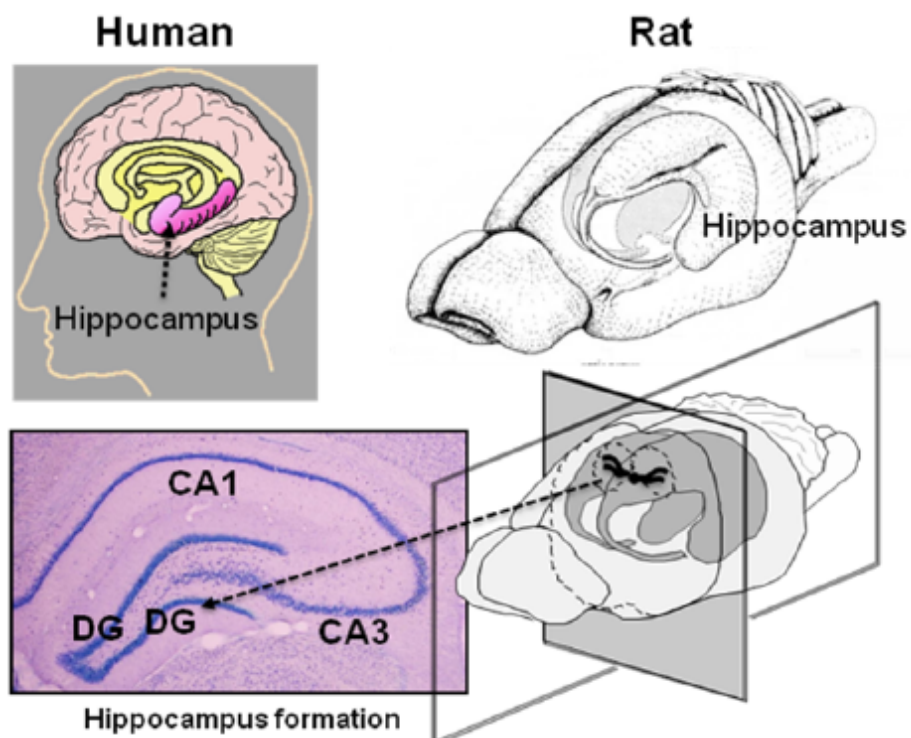


Figure 3: Hippocampus location (human and rat) and simplified anatomy (rat)

This hippocampus contains different subfields, including the dentate gyrus (DG) and the ammon's horn (cornu ammonis, CA) itself subdivided into CA1, 2, 3. The CA1 and CA3 regions are connected by the Schaffer collaterals, being CA3 pyramidal neurons projecting their axons to CA1. This Schaffer collateral-commissural pathway has been investigated in studies involving synaptic plasticity (Collingridge et al., 1983 ; Schiess et al., 2010)

The hippocampus in rodents has been studied extensively in order to get further insights into the development of neurological mechanisms such as memory in humans, as various similarities have been conserved throughout time and evolution (Clark and Squire, 2013). It

has to be noted, though, that discoveries involving rodents are not directly transferrable to humans, but can be of great help in the establishment of models about gene expression, for example. The reason behind this is that, while being similar to a certain extent (genetic traits have been conserved throughout evolution), humans and rodents do not have the exact same metabolism.

3) Neurotransmissions

Excitatory and inhibitory neurotransmissions

In the central nervous system, neurons are interconnected to form networks that can pass on information in the shape of electric signals. These connections are called synapses (Fig. 4) and can be of two forms : excitatory and inhibitory. The balance between the two is crucial in normal brain function in order to create synchronous oscillatory activity. In some epilepsy cases, not related to AD, the presence of an imbalance between excitatory and inhibitory neurotransmissions in the hippocampus has been shown (Bonansco and Fuenzalida, 2016), which demonstrates the need for a finely tuned balance.

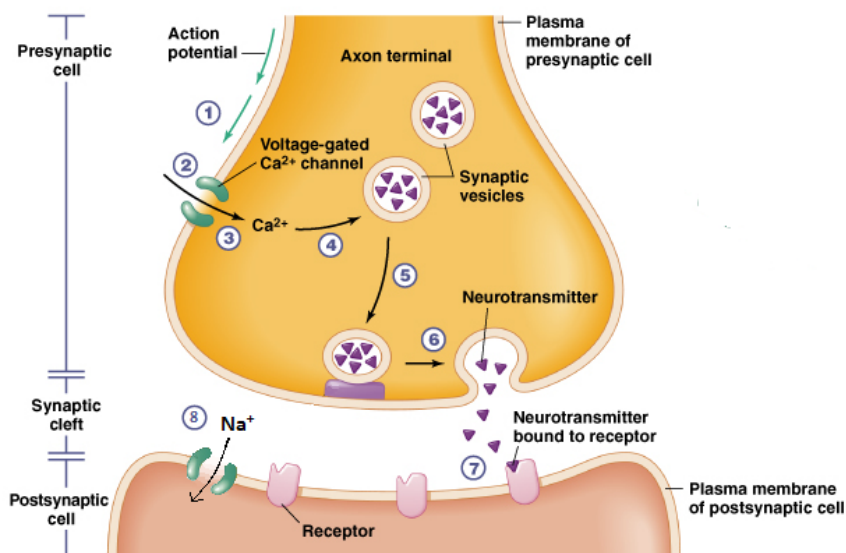


Figure 4: Synapse

- 1) An action potential coming from the presynaptic cell, causing membrane depolarization
 - 2) In the presence of a depolarization, voltage-gated calcium ions channels open
 - 3) Calcium ions influx to the cell
 - 4) Calcium ions interact with proteins on the membrane of the synaptic vesicles
 - 5) The synaptic vesicle membrane fuses with the presynaptic button membrane
 - 6) Neurotransmitters are released and diffuse in the synaptic cleft
 - 7) Neurotransmitters bind to their receptors
 - 8) Receptor binding of the neurotransmitters leads to the opening of sodium channel which in turn leads to the depolarization of the membrane and formation of a new action potential in the postsynaptic cell
- Taken from <http://biology4alevel.blogspot.com/2016/06/122-synapses.html>

Excitatory neurotransmission

Glutamate is the main neurotransmitter in excitatory synapses. When released in the synaptic cleft, it will bind to post-synaptic metabotropic (mGluR1-8) and ionotropic (iGluR) receptors leading to, for the former, the activation of intracellular signalling cascades and, for the latter, ion channels opening.

These ionotropic receptors are ligand-gated ion channels and are responsible for the majority of the excitatory neurotransmissions as well as playing an important role in synaptic plasticity, being the ability of neurons to form dynamic networks that gain or lose strength over time depending on how they are solicited. These receptors are the Kainate, AMPA (named after the synthetic agonist α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and N-methyl-D-aspartate (NMDA) receptors, which will be detailed further. When glutamate binds to its receptors present on the post-synaptic neuron, ion-channels open and let ions such as sodium (Na^+) and calcium (Ca^{++}) flow through the membrane leading to its depolarization. Once this depolarization goes beyond a certain threshold, a new action potential is generated, in turn the post-synaptic neuron « fires » and information is passed on. There are two types of action potential, depending on the ion-specific channel considered. The voltage gated sodium channel are responsible for fast acting and fast decaying action potential, that usually last for less than a millisecond while the calcium channels generate action potentials that can last up to 100 milliseconds or more.

Inhibitory neurotransmission

Excitatory neurotransmissions are connected to and regulated by inhibitory neurotransmissions (Van vreeswijk and Sompolinsky, 1998). The main neurotransmitter for these connections is γ -amino butyric acid (GABA) and, as with glutamate, will bind to ionotropic and metabotropic receptors when released in the synaptic cleft. This binding will enable chloride ions (Cl^-) to pass through the cell membrane down their concentration gradient which in turn leads to a massive entrance of negative charges, generating a hyperpolarization of the neuron's membrane (Farrant and kaila, 2007).

Synaptophysin is an integral pre-synaptic membrane glycoprotein localized to synaptic vesicles. It has been shown that synaptophysin apparently plays a role in the regulation of activity-dependent synaptic formation (Tarsa and Goda, 2002) where it acts at the moment of the vesicle-presynaptic membrane fusion. Accordingly, this protein is commonly used as sensitive marker for synaptic terminal regions. Furthermore, it has been reported that synaptophysin concentrations are decreased in some areas of AD patients' brain.

Post synaptic density protein 95 (PSD95) is a protein apparently involved in the formation of postsynaptic clustering of ion channels and receptors including NMDAR and AMPAR (Sheng and Sala, 2001). As its name indicates, it is prominently, if not exclusively located at the post synaptic region of neurons (Hunt et al., 1996). According to Meyer et al. (2014), PSD95 is key in synaptic plasticity and in processes involved during long-term potentiation (Synaptic plasticity and long-term potentiation are detailed further down).

These pre- and post-synaptic proteins are mentioned here above due to the fact that in AD patients, it has been shown that a decrease in the expression of both synaptophysin and PSD95 appear in areas of the brain such as temporal and frontal cortex as well as the hippocampus (Masliah et al., 1994 ; Love et al., 2006 ; Sultana et al., 2010). This decrease in the expression of both protein reflects synaptic disruption (Yuki et al., 2015), and Cullen et al. stated, in 1997, that the temporal and frontal cortices are in fact the regions where neuronal loss is most prominent.

Synaptic plasticity

« Neurons that fire together, wire together ».

In the 1949 book « Organization of behaviour : a neuropsychological theory », Donald O. Hebb states what has become a widespread consensus : learning changes the brain cellular structure. From this derives the concept of synaptic plasticity, which can be described as the ability of synapses to gain or lose in strength or potential intensity following increases or decreases in their activity, respectively. The more neural networks are solicited,

the more they will be effective. The major mechanisms linked to synaptic plasticity are called long-term potentiation (LTP), and its counterpart long-term depression (LTD) (Fitzjohnnet al., 2008). LTP is characterized by an increase in excitatory post-synaptic potential, meaning an increase in synaptic strength, which is thought to be a cellular manifestation of the learning and memory processes. (Bredt and Nicoll, 2003 ; Malenka and Bear, 2003 ; Malinow and Malenka, 2002). The synaptic strength depends on two mechanisms : one related to the amount of neurotransmitter to be released by the presynaptic neuron in the synaptic cleft and, on the other hand, the amount and function of receptors to these neurotransmitters to be present at the postsynaptic neuron's membrane. The two main receptors to be discussed here are the ionotropic AMPA and NMDA receptors that take part in the triggering of LTP.

AMPA Receptors

AMPA receptors (AMPA) are mostly tetramers composed of four types of glutamate-binding subunits (GluA1-4) encoded by the GRIA1-4 genes. They usually appear as heterotetramer consisting of a dimer of GluA2 and a dimer of either of GluA1,3 or 4 (Mayer and Greger) (see Fig. 5) but in the mature hippocampal region (in the excitatory CA3-CA1 synapses), the common form appear to comprise GluA1/GluA2 or GluA3 subunits (Holman et al., 2007). The difference in subunit composition has an impact on the properties of the tetramer, even though they are quite similar to one another. The presence or absence of GluA2 influences greatly the behaviour of the tetramer. For instance, the presence of GluA2 is linked to a decrease in the opening probability, channel conductance and Ca^{2+} permeability of the receptor. (Swanson et al., 1997 ; Oh and Derkach, 2005 ; Burnashev et al., 1992 ; Burnashev, 2005-67). AMPARs are the main responsible for fast excitatory neurotransmissions in the mammalian brain.

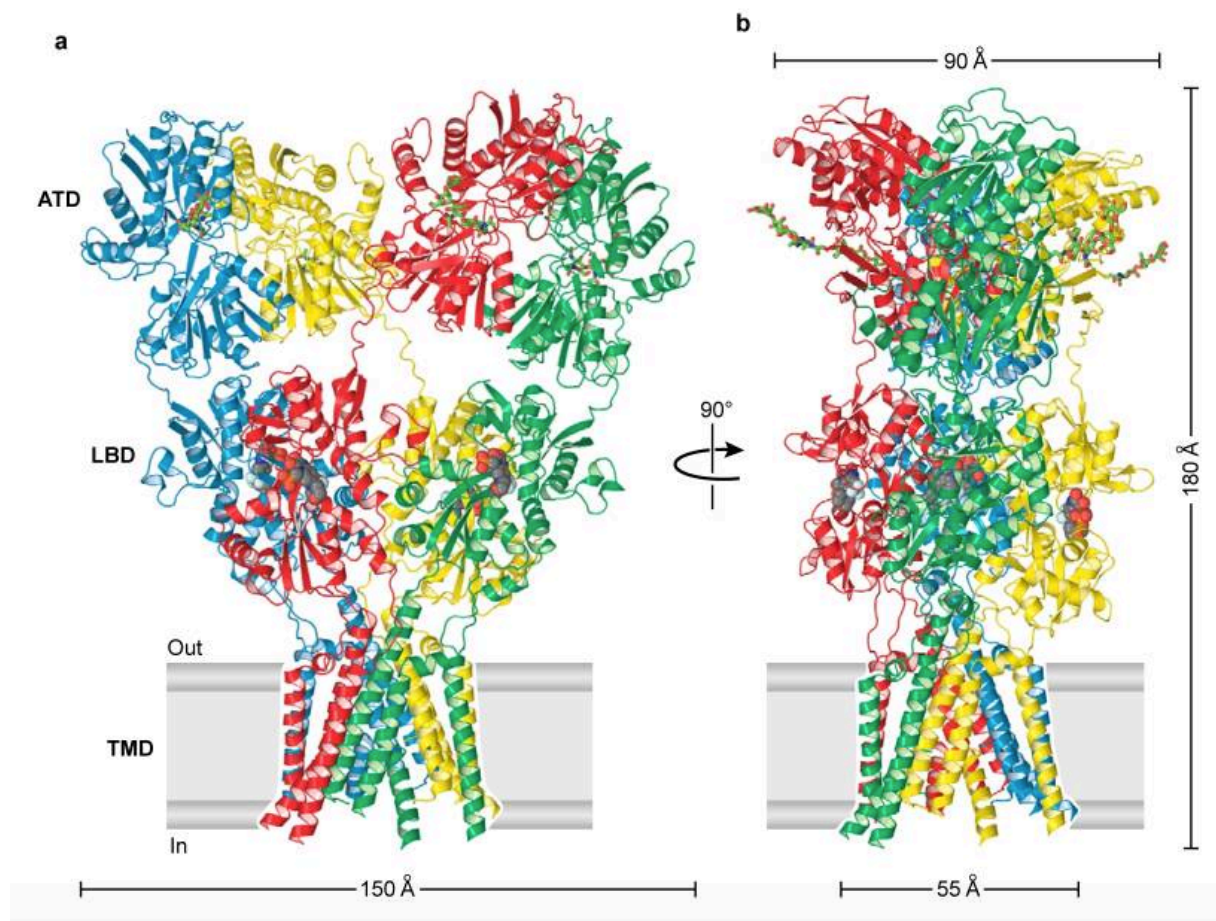


Figure 5: AMPA receptor tridimensionnal structure. a) View of the wide face of the receptor. b) View of the narrow face of the receptor, rotated 90° from a).

Each subunit is represented with a different color

ATD : Amino terminal domain

LBD : Ligand binding domain

TMD : Transmembrane domain

From Sobolevsky et al., 2009

NMDA Receptors

As stated before, NMDA receptors (NMDARs) are crucial for the establishment of synaptic plasticity. These receptors are activated when glutamate and glycine are bound to them, allowing an influx of positively charged ions through the neuron's membrane (Furukawa 2005). A key feature to these NMDA receptors is the presence of a voltage-sensitive block by an extracellular magnesium ion (Mg^{2+}) which can be dislodged when the postsynaptic neuron is depolarized (only past a certain threshold). When glutamate and co-agonist glycine (or D-serine) bind to the receptor as well as the depolarization being high enough, calcium ions (Ca^{2+}) are allowed to flow through the channel inside the cell regulating in turn the synaptic strength (Fig. 6).

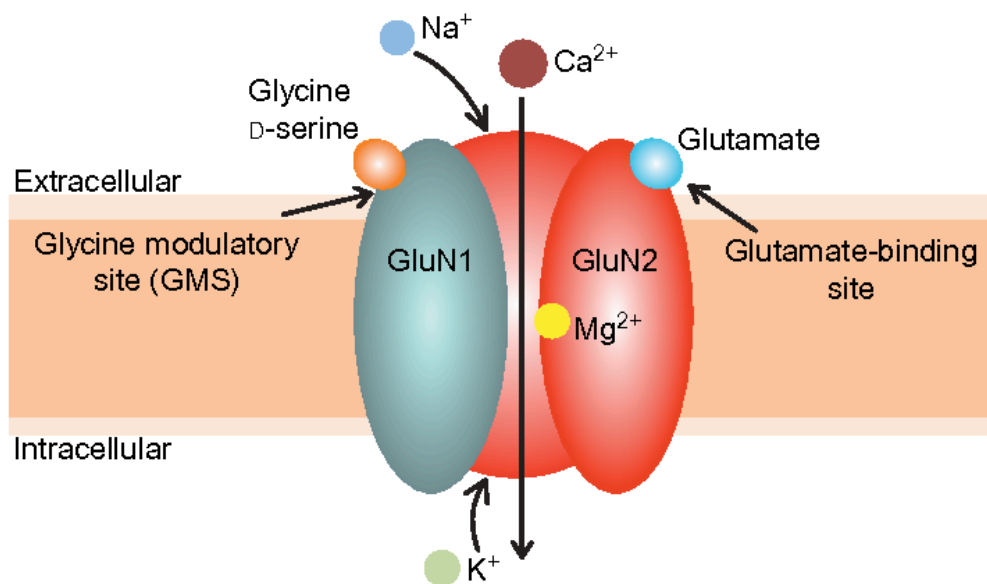


Figure 6 : Structure of the NMDA receptor. The conventional NMDAR ion channel is heterotetrameric, consisting of two GluN1 and two GluN2 subunits. These receptors act as molecular coincidence detectors, as in addition to the binding of its agonist glutamate to the GluN2 subunit, activation of the NMDAR requires (1) postsynaptic depolarization, which relieves the Mg^{2+} blockade of the channel and (2) either glycine or D-serine must be bound at the glycine modulatory site (GMS) on the GluN1 subunit. Upon NMDAR channel opening, calcium (Ca^{2+}) and sodium (Na^{+}) enter the neuron, while potassium (K^{+}) exits the neuron. (Figure taken from Balu, 2016)

NMDARs subunits (GluN1, GluN2A-D and GluN3A-B) are encoded by three families of genes : GRIN1, GRIN2 and GRIN3 respectively. Different studies (crystallographic, biochemical and electrophysiological) pointed out that NMDARs are present in the form of two GluN1 subunits assembling two GluN2 subunits (Cull-Candy and Leszkiewicz, 2004 ; Furukawa et al., 2005), forming an heterotetramer. The spatial and temporal expression of GluN2A and GluN2B differ, as GluN2A shows an increase as neurons mature (Cull-Candy et al., 2001) and on the other hand GluN2B shows the opposite. Moreover, GluN2B-containing receptors present slow kinetics while GluN2A-containing receptors exhibit fast kinetics (Cull-Candy and Leszkiewicz, 2004). Consequently, it is thought that the GluN2B/GluN2A ratio plays a role in the developmental switch in decay time of NMDA receptors-mediated currents (Cull-Candy et al., 2001).

Different studies have shown that a certain group of transcription factors, the nuclear receptors, are related to excitatory neurotransmissions (Roy et al., 2013).

4) Nuclear receptors

Retinoid X receptors (RXRs), along with liver X receptors (LXRs), peroxysome proliferator-activated receptors (PPARs), retinoic acid receptors (RARs), thyroid hormone and vitamin D receptors form together a family of nuclear receptors (NRs) present in metazoans, but not in plants, fungi or protists (Escriva, 1998). They present the ability to directly bind to DNA and regulate gene expression by binding to sequence-specific elements on the promoter regions (Szanto et al., 2004; Boergesen et al., 2012) and, therefore, can be referred to as transcription factors (Olefsky, 2001). The superfamily of NRs contains 4 types of receptors, depending on their subcellular location and sequences they bind to, called hormone response elements (HREs). On the structural point of view, NRs possess different domains, as follows:

- N-terminal ligand-independent activation domain, for coactivator involvement.
- C-terminal ligand-binding domain.
- DNA-binding domain.

Type II nuclear receptors LXRs and PPARs have the particularity of forming obligatory heterodimers with RXRs, in a permissive manner (Evans and Mangelsdorf, 2014) as opposed to heterodimers formed with vitamin-D receptor or thyroid receptor. These type II NRs are retained in the nucleus, regardless of their ligand binding status (Klinge, 1997; Amoutzias, 2007).

RXR

The first RXR isotype to be discovered, and referred to as RXR α , was initially described as an orphan receptor, having no endogenous ligand (Mangelsdorf et al., 1990). The term RXR was chosen due to the fact that all-*trans*-retinoic acid could activate RXR α . Later on, it was found that 9-*cis*-retinoic acid is a ligand showing high affinity for not only RXR α , but for the other two later discovered subtypes as well (RXR β and RXR γ) (Rowe et al., 1991; Yu et al., 1991; Heyman et al., 1992; Leid et al., 1992; Levin et al., 1992; Mangelsdorf et al., 1992). Upon ligand binding, RXR will stimulate the transcription of ATP-binding cassette sub-family A member 1 (ABCA1) which is, along with the lipidated apolipoprotein E (ApoE), involved in cholesterol transport in the brain (Liang et al. 2004 ; Zhao et al. 2014).

Other synthetic agonists (called retinoids) can also activate RXRs.

Bexarotene (with Targretin® as its commercial name) is an anti-neoplastic drug, used in the treatment against cutaneous T cell lymphoma (Gniadecki et al., 2007). Bexarotene is a third-generation retinoid agonist for RXRs. Therefore, bexarotene has an effect on lipid homeostasis through not only ApoE and ABCA1 transcription stimulation (stated before), but also by activating the transcription of the sterol regulatory element-binding protein 1 (SREBP1) encoding gene, resulting in hypertriglyceridemia (Baranowski, 2008). In addition, different studies have shown beneficial effects of the RXR agonist on cognitive behaviour in AD mouse models. (Cramer et al., 2012 ; Boehm-Cagan and Michaelson, 2014) Furthermore, biological markers as well as cognition improvements through treatment with bexarotene have been reported on a patient suffering from mild Alzheimer's (Pierrot et al., 2015). Depending on the heterodimerization partner of RXR, different types of response elements are recognized (Glass, 1994) and different metabolic effects will arise, which will be detailed further.

LXR

Liver X receptors, classified into subfamily 1 of the NRs (along with thyroid receptor) are subdivided into two isoforms, LXR α and LXR β . The former is expressed in the liver, intestine, kidney, spleen, and adipose tissue, and the latter is ubiquitously expressed, but on lower levels (Auboeuf et al., 1997 ; Repa and Mangelsdorf, 2000) though more widely in the brain, in the hippocampus in particular (Bookout et al., 2006 ; Kainu et al., 1996). Formerly identified as an orphan receptor, it was later discovered that oxysterols, main forms of hydroxylated cholesterol, are endogenous ligands (Janowski, 1996 ; Lehmann et al., 1997 ; Song, 2000). These receptors play a critical role in whole body cholesterol homeostasis and also present potent anti-inflammatory actions (Lehmann et al., 1997). Upon activation, target genes involved in the cholesterol metabolism are transcribed, under LXR control. The product of these genes are ABCA1 and ApoE which, as stated before, are the main cholesterol carriers in the brain, as well as the sterol regulatory element-binding protein 1 (SREBP1) (Gan et al., 2001 ; Langmann et al., 2005). SREBP1 is a transcription factor and has a role in fatty acid, lipid and cholesterol production, and is regulated by insulin and cellular

sterol levels. It also regulates glucose metabolism by acting on certain genes. (Ferré and Foufelle, 2010 ; Eberlé et al., 2004)

PPAR

Peroxisome proliferator-activated receptors (PPARs), along with RXRs and LXRs act as regulators of the lipid metabolism via transactivation of genes involved in the processing of fats and lipids. At first, PPARs were classified as orphan receptors but later on, endogenous ligands such as fatty acids and eicosanoids coming from dietary lipids were identified. To this day, three different isoforms differently distributed among tissues of PPARs are known, PPAR α , PPAR β/δ and PPAR γ .

The first isoform to be discovered, PPAR α , has been shown to regulate fatty acid oxidation by controlling the expression of acyl-CoA oxidase, an enzyme involved in the β -oxidation of fatty acids and specific to peroxysomes (Osumi et al., 1980). Accordingly, PPAR α is a target for compounds prescribed to patients suffering from hypercholesterolemia (e.g. hypolipidemic fibrates) (Staels et al., 1998).

As expected, PPAR α is substantially expressed in liver tissues as well as in heart and kidney but at low level in the brain (Kliwer et al., 1994 ; Tugwood et al., 1998).

Interestingly, though the hippocampus does not yield energy from fat metabolism, it was shown that PPAR α is expressed in different regions of the structure in astrocytes as well as hippocampal neurons.

PPAR β/δ plays a role in fatty acid oxidation in liver, kidney, adipose tissue and skeletal muscle where it is the most abundant isoform while it is expressed ubiquitously in all tissues. It is the dominant isoform in rat brain as well and is expressed in all cell types.

PPARs activation is able to increase BDNF expression, as well as expression of AMPA and NMDA receptors in hippocampal neuronal cells. Interestingly, PPAR α agonists specifically induce the activation of Creb promoter in hippocampal neurons from wild type, but not from PPAR α null mice.

II) Objectives

Bexarotene has been shown to improve cognition in AD mouse models and, in some cases, impact positively some AD patients.

The general aim was to study the effect of bexarotene after 24 hours on primary cultures of rat hippocampal neurons through measurement of relative expression of genes recognized as key in the development of synaptic plasticity, crucial for the establishment of memory processes. This assessment was performed using retrotranscription quantitative polymerase chain reaction (RT-qPCR), in order to measure relative quantities of messenger RNA, and Western-blotting, to measure relative quantities of proteins.

III) Material et methods

1) Primary Culture of hippocampal neurons

1.1. *Principle*

A primary culture consists of the harvest of cells coming directly from a living tissue and setting them in equal quantities in multi-well plates, in the appropriate medium. In our case, the cells are hippocampal neurons taken from brains of Wistar rat embryos after 17 to 18 days of gestation

1.2. *Protocol*

1.2.1. Plates pre-incubation

In order to insure the adherence of the neurons to the plate, a treatment using poly-L-lysine (10 mg/ml) is carried out prior to the culture. This has the effect of neutralizing the anionic charges present on the plastic surface, which prevents the neurons to stick effectively. This pre-incubation is carried out during at least 2 hours, before removing the lysine and replacing it by PBS 1X (composition detailed in annex)

1.2.2. Cells harvest and culture

The pregnant rat is put to sleep and euthanized using carbon dioxide. This being done, the animal's abdomen is disinfected with ethanol 75%. With the help of dissection tools, a cut is performed on the lower part of the abdomen in order to reach the uterus and the embryos' sack, from which the embryos string is removed from the womb. Each individual embryo is isolated and put in PBS 1x (see composition in the annexes). While in sterile conditions, the skull is cut open and the brain extracted and put in a Petri dish containing CMF-HBSS (composition detailed in annex) for the dissection with binoculars.

The two hemispheres are separated and the meninges removed, and then the hippocampi are isolated and placed in a 15 ml falcon tube containing 4,5 ml of CMF-HBSS and 0,5 ml of trypsin 2,5% and put at 37°C for 15 minutes. This being done, the supernatant is removed

with good care not to touch the hippocampi that are then washed twice using CMF-HBSS in order to remove any remaining trypsin.

The hippocampi are mechanically dissociated using Pasteur pipets, to fully separate the neurons (no chunks of tissue can be left in solution).

This solution is centrifugated for 5 minutes at room temperature (RT) at 900g, the supernatant is removed and neuronal plating medium (NPM, composition detailed in annex) is added. The cells are counted using a Bürker counting chamber then are put in the lysine treated multi-well plates (around 600.000 cells per well in a 24 well-plate). After 3 to 4 hours at 37°C, the NPM is replaced by B27 supplemented neurobasal medium (composition detailed in annex) and the plate is put back at 37°C. The medium must be changed every 2 to 3 days.

The neurons are put in the incubator at 37°C for 13-14 days.

1.2.2.3. Treatment

Bexarotene (100 mM) is added to fresh medium 24 hours before harvest to half of the culture wells (the medium of the other half is replaced as well).

2) RNA extraction

2.1. Principle

The extraction of RNA is performed using a chaotropic agent (guanidine thiocyanate), which has the effect of weakening the hydrophobic effect and cell lysis, destroying the three-dimensional structure of proteins (including RNAses and DNAses) and DNA precipitation. Using chloroform and high-speed centrifugation, the DNA and proteins are separated from the RNA, being in organic and aqueous phases, respectively.

2.2. Protocol

The neurons are lysed using 500 µL of Tripure Isolation Reagent during 5 minutes at room temperature (RT). After that, 100 µL of chloroform are added to the solution which is set for 10 to 15 minutes at RT before centrifugation at 12.000 g, 4°C for 15 minutes. The aqueous phase is put in 250 µL of isopropanol and glycogen (to ease precipitation) and after 5 to 10 minutes of incubation, is centrifugated again at 12.000 g, 4°C for 10 minutes. The

pellet formed is washed using cold (-20°C) ethanol 75% and a last centrifugation is carried out at 7.500 g, 4°C for 5 minutes before being left to dry. The pellet is finally solubilized in diethyl pyrocarbonate (DEPC) treated water.

The quantity and purity of harvested RNA is measured using a nanodrop spectrophotometer, and the volume of each sample is adjusted using DEPC-treated water in order to reach a final concentration of 1 µg / 10 µL. The samples are stored at -80°C to prevent RNA degradation.

3) Retrotranscription

3.1. Principle

Retrotranscription allows the synthesis of complementary DNA (cDNA) from extracted messenger RNA (mRNA). This synthesis is performed using the iScript retrotranscription kit that includes the iScript Reaction Mix, the iScript Reverse Transcriptase and nuclease-free water.

3.2. Protocol

Each sample containing 1 µg of RNA (per 10 µL) is placed in a tube containing 1 µL of reverse transcriptase, 4 µL of reaction mix containing the oligo-dT's, random nucleotides hexamers and dNTP. Using DEPC-treated water, the final volume is adjusted to 20 µL.

Using a thermocycler, the three different steps are achieved as follows :

- Primers' hybridation : 5 minutes at 25°C
- cDNA elongation : 30 minutes at 42°C
- RNA degradation : 5 minutes at 80°C

Once the cDNA synthesis is finished, the samples' concentration is set to 100 ng/10 µL first then to 8 ng/10 µL. the remaining fractions at 100 ng/10 µL are pooled together and will be used to establish a standard curve during the quantitative Polymerase Chain Reaction (qPCR) performed afterwards.

4) Quantitative polymerase chain reaction (qPCR)

4.1. Principle

4.1.1. PCR

The analysis of cDNA using qPCR allows one to assess quantitatively the mRNA expressed related to a certain gene in different experimental conditions (e.g. the presence of Bexarotene in the culture medium). In our case, qPCR is used to compare the expression levels of different genes in the two experimental conditions. The quantification happens thanks to the use of a fluorescent dye called SYBR Green that has a high affinity for the minor groove of the double helix, henceforth binding to double stranded DNA and, when bound, will absorb blue light and emit green light (The dye does emit a low fluorescence when in solution, the binding enhances the phenomenon).

FIGURE

Three different steps are performed during the process of qPCR (as in retrotranscription).

a) Denaturation

This step consists of the separation of double stranded DNA by increasing the temperature to a set point (past the melting point but not so high as to avoid DNA degradation).

b) Hybridization

Each cDNA fragment one is willing to amplify requires the use of primers that are short sequences of nucleotides (in our case around 20 nucleotides) that are present in the original sequence at the beginning of the gene sequence in both strands (forward and reverse primers). The designed primers (synthesized by the firm Sigma-Aldrich) should have a percentage of G-C of around 50% and a melting temperature of roughly 60°C in order to insure a certain level of efficiency. Furthermore, one should pay attention to the sequence chosen for the design and make sure that there won't be palindromic portions that could self anneal during the hybridation step and prevent the PCR from running correctly.

c) DNA elongation

This phenomenon is performed through the use of an enzyme : the Taq Polymerase (*Thermophilus aquaticus* being the organism from which the enzyme was isolated).

This enzyme adds nucleotides complementarily to the strand it is attached on, starting with the primers previously designed and present in the reaction mix during the first cycle. As the second one starts, the generated amplicons serve as primers themselves and make this process follow an exponential profile. During this elongation, double stranded cDNA is synthesized all along and, as stated before, the SYBR Green dye will bind to it and will emit green light that is measured for the quantification.

4.1.2. Quantification

The quantification is based on the fluorescence emission. Past a certain number of amplification cycles (called cycle threshold, Ct) the emission soars above the background noise level. This Ct value is inversely proportional to the quantity of cDNA (gene assessed) present at the beginning of the first cycle, meaning that a high Ct value is linked to a low genetic material concentration in the sample.

Using a reference gene as well as a reference experimental condition, one is allowed to quantify the assessed gene expression in a relative manner. In our case the gene coding for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as a reference gene as it is expressed in quite high and constant quantity in the different experimental conditions.

Once the Ct values for each sample are acquired, different steps are needed to be performed in order to have comparable data. These steps are as follows :

- Normalization relative to the reference gene.

$$Ct_{\text{target gene}} - Ct_{\text{GAPDH}} = \Delta Ct_{\text{sample}}$$

- Normalization relative to the reference condition, being the neurons that were not treated with bexarotene for 24 hours.

$$\Delta Ct_{\text{sample}} - \Delta Ct_{\text{reference condition}} = \Delta \Delta Ct$$

- Determination of the difference in copies of mRNA (n) expressed of the assessed gene.

$$n = 2^{-\Delta\Delta Ct}$$

4.2. Protocol

For each sample analysed, 8 ng of cDNA are added to 12,5 µL of iQ SYBR Green Supermix (annex) and 625 nM of forward and reverse primers (annex). Each sample is tested three times (technical triplicates) and each of the 4 standard points in duplicates. The standard points are obtained via the serial dilutions (4 fold) starting from the pooled cDNA at 100 ng/10 µL up to 1,56 ng/10 µL.

The different cycles performed are as follows :

- 1 cycle at 95°C for 3 minutes
- 40 cycles :
 - * 30 seconds at 95°C
 - * 45 seconds at 60°C
 - * 15 seconds at 79°C
- 1 cycle at 55°C for 30 seconds

5) Western blotting

Western blotting is a technique used to measure the expression level of certain proteins of interest present in cells' lysate. This technique is performed via three steps being :

- Electrophoresis of the proteins on a SDS-PAGE gel
- Transfer of the proteins from the gel to a nitrocellulose membrane
- Immunoassay of the proteins using specific antibodies

When harvesting the neurons at the end of the primary culture, the culture medium is recovered and the cells are washed twice and then put in solution using PBS 1x, centrifugated at 16,000 g for 2 minutes at 4°C. The supernatant is then discarded and 30 µL of lysis buffer (Laemmli 2X, composition detailed in annex) are added. The resulting solution is sonicated then centrifugated at room temperature for 2 minutes. This is the starting material for the different procedures needed in order to achieve a western blotting analysis.

5.1. Protein assay

5.1.1. Principle

The protein assays consists of measuring the total amount of proteins present in the sample. This needs to be done as the electrophoresis requires one to put an equal amount of proteins for each sample in order to retrieve comparable results at the end.

The measure is performed via colorimetry, using the BCA (bicinchonic acid) protein assay kit (Pierce). The kit works as follows :

Two reactants (A and B respectively) are provided, bicinchonic acid (BCA) and copper II sulfate. When put in solution with both reactants, the proteins will reduce the copper II ions to copper I ions, which in turn will chelate two molecules of bicinchonic acid, forming a colored complex. The absorbance of said complex is measured at $\lambda=562$ nm using a spectrophotometer. A standard curve is established with the kit using Bovine Serum Albumin (BSA, Thermo Fischer Scientific), allowing quantification by extrapolation of the samples' proteins contents.

5.1.2. Protocol

a) Standard curve

The standard curve is established with a serial dilution (2 fold) using a stock solution of Bovine Serum Albumin (BSA, 2 mg/mL), the 6 points measured start from a concentration of 1000 down to 31,25 $\mu\text{g/mL}$. The final volume measured using the colorimeter is 50 μL , and a blank is performed using 50 μL of distilled water.

b) Sample preparation

Each sample is diluted 10 times (5 μL of sample added to 45 μL of distilled water). A blank is performed using the lysis buffer (Laemmli 2X) diluted 10 times as well

c) Measures

1 mL of BCA protein assay reaction mixture (composition annex) is added to each of the standard points and samples, the resulting solutions are then put for 30 minutes at 37°C for incubation. Finally, the absorbance is measured and the amount of proteins in each sample is acquired with a spectrophotometer.

5.2. Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE)

5.2.1. Principle

Electrophoresis using polyacrylamide gel and SDS allows to separate proteins based on their size. SDS is a detergent that denaturates proteins by removing the non-covalent bonds as well giving a constant negative charge density, meaning that long peptides will present a higher negative charges count.

Using this property, an electric field is applied longitudinally to the gel in order to make the now negatively charged proteins move towards the positive electrode, relatively to their total charge (and so, to their size).

The polyacrylamide gel, on the other hand, can be seen as a molecular sieve. The branched polymer density can be controlled and is used to separate the proteins as well by preventing them from passing through the pores of the polymer in the separating – resolving – part of the gel. In Our case, gels presenting a gradient in density were used to resolve our harvested proteins.

5.2.2. Protocol

An equal amount of proteins (20 µg) is put in solution with the loading buffer (LAEMMLI 2X or 4X) before being put in a heating block at 95°C for 5 minutes, in order to denaturate said proteins. The presence of β-mercaptoethanol in the buffer participates in the process by reducing the disulfur bonds between the amino-acids of the different proteins.

The NuPAGE Novex Bis-Tris 4-12% (from Invitrogen) pre-made gels have been used to conduct the analysis. The gels are set in the Xcell SureLock Mini-Cell (electrophoresis cell) containing the MES/SDS running buffer with which the different wells are washed prior to proteins loading. The use of a standard proteins ladder (Novex Sharp), loaded in the first well is necessary to have a reference as to the size-relative migration profile. Once the proteins are loaded, the lid is put on the cell, closing the circuit. The migration is operated for 2 hours under a 90 Volts electric tension at room temperature.

5.3. Nitrocellulose membrane transfer

5.3.1. Principle

Once the migration completed, the resolved proteins are then transferred to a protein-binding nitrocellulose membrane. Polyvinylidene difluoride (PVDF) membranes can

be an alternative. These membranes bind protein non-selectively via hydrophobic and ionic interactions. The aim of the transfer is to put the proteins on a thin layer, allowing more contact surface for the staining that will follow. Contrarily to the SDS-PAGE process, the electric field is applied perpendicularly to the aligned gel and membrane to ensure a one directional transfer. The transfer is conducted in a specific buffer, which contains methanol, in order to remove the SDS coating the proteins and thus ensuring protein binding to the membrane.

5.3.2. Protocol

The different components necessary to conduct the transfer (sponges, filter papers and nitrocellulose membrane) are first soaked in the migration buffer (composition detailed in annex) and are stripped from any residual air. The transfer assembly is conducted from the bottom up, starting at the cathode. The different components are stacked up as shown in figure

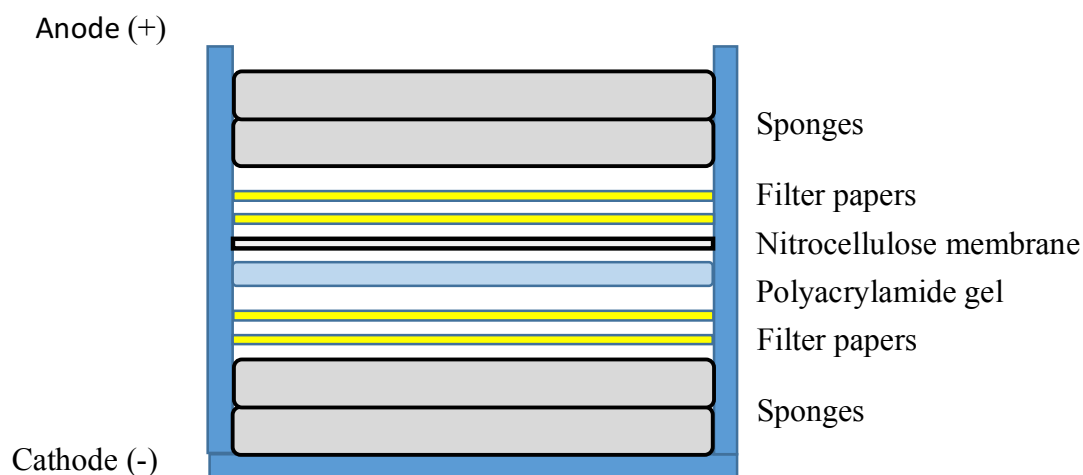


Figure 7: Membrane transfer cell assembly

Once all the components are in place and rid of air (most critical point being between the gel and the membrane), the transfer cell is closed and hereby compressed and filled with the transfer buffer. An electric tension of 30 volts is applied for 2 hours at room temperature.

5.4. *Total protein staining*

5.4.1. Principle

After completion of the protein transfer onto the nitrocellulose membrane, said membrane is put for in a solution containing acetic acid and Ponceau red (composition detailed in annex). This solution will reversibly color the protein, making them visible for the naked eye. This transient coloration allows one to visualize the quality and homogeneity of transfer as well as, if needed, to cut the membrane in strips that will be incubated in different antibodies for the following immunoassay.

5.4.2. Protocole

The membrane is put in the Ponceau red solution for 5 minutes at room temperature under gentle agitation. Afterwards, the membrane is washed twice with PBS 1X to remove the excess of Ponceau red not adhering to the proteins. If needed, the membrane is cut along the molecular weight ladder.

5.5. *Immunostaining*

5.5.1. Principle

The final step of the western blotting analysis is the immunostaining. The aim is to show the presence of a certain protein of interest on the nitrocellulose membrane. This is carried out via the use of specific antibodies. First, the membrane is incubated in a solution of blocking agent in order to prevent the aspecific binding of the primary antibodies on the sites present on the membrane, leading to an increase of the background signal once the immunoassay is completed. The membrane is then put in a solution containing the primary antibodies specific to the tested protein (see table X), and then in a solution containing secondary antibodies, paired with an enzyme (in our case the horseradish peroxidase) and directed against the primary ones. This enzyme, when put in presence of hydrogen peroxide and luminol, will generate a chemoluminescent compound. The amount of this compound is proportional to the amount of enzyme and thus, the amount of tested protein present on the membrane. This light emission is then used to impress a photosensitive film and later be quantified.

5.5.2. Protocole

Following the total protein staining and membrane cutting, said membrane is put for an hour at room temperature in a solution of PBS 1X containing 0,05% of Tween20, a detergent, and powdered milk (5%) under mild agitation. Afterwards, the membrane is washed twice in PBS 1X –Tween 0,05% for 5 minutes to remove the excess milk. Then, the incubation with the primary antibodies is carried out overnight at 4°C.

Primary antibodies	Type and production species	Dilution factor
GluN2A	Monoclonal, rabbit	1/250
GluN2B	Monoclonal, mouse	1/500
GluA1	Monoclonal, rabbit	1/500
GluA2	Monoclonal, mouse	1/1000
α -Tubulin	Monoclonal, mouse	1/4000
Synaptophysin	Monoclonal, rabbit	1/1000
ABCA1	Monoclonal, mouse	1/1000
PSD95	Monoclonal, rabbit	1/1000

The following day, the membrane is washed twice for 10 minutes with PBS 1X-Tween 0,05% (in order to remove unbound antibodies) before being put for incubation with the secondary antibodies (see table X) for an hour under mild agitation at room temperature.

Secondary antibodies	Type and production species	Dilution
Anti-mouse	Polyclonal, sheep	1/10000
Anti-rabbit	Polyclonal, donkey	1/10000

Afterwards, the membrane is washed twice for 10 minutes under strong agitation in PBS 1X - Tween 0,05%. Then, the membrane is put in presence of luminol and hydrogen peroxyde (using the western lightning plus ECL kit) for a minute in order to carry out the reaction involving the antibody-bound peroxydase, before exposure to the photosensitive film. Different exposure times are performed in order to make sure each protein can then be quantified properly, taking into account their differences in concentration.

Statistical Analysis

The results obtained through the use of the different methods described above have been statistically analysed in order to confirm or refute the relevance of the various patterns found in the gathered data. The tool used to carry out these analyses is the software GraphPad Prism.

Shapiro-Wilk's w-test

Principle

The first test carried out on the processed data is the Shapiro-Wilk normality test. Its goal is to determine if a given sample comes from a normally distributed population or at least can be considered as not significantly deviating from normality.

The null hypothesis H0 is that the population is indeed normally distributed. If the p-value is found to be less than the chosen alpha-level (in our case 0,05), the null hypothesis is rejected and the data cannot, thus, be considered to come from a normally distributed population. On the other hand, if the p-value is greater than the alpha-level, the null hypothesis cannot be rejected and the data can be assumed to come from a population which does not deviate from normality so as to significantly impact the drawn results.

Theory

Here follows the different formulas necessary to conduct the Shapiro-Wilk normality test.

The test statistic is :

$$W = \frac{\left(\sum_{i=1}^n a_i x_{(i)}\right)^2}{\sum_{i=1}^n (x_i - \bar{x})^2}$$

With :

- x_i being the ordered random sample values,

- $\bar{x} = \frac{x_1 + \dots + x_n}{n}$ being the sample mean,

- a_i being constants generated from the means, covariances and variances of the sample from a normally distributed sample

- $(a_i, \dots, a_n) = \frac{m^T V^{-1}}{(m^T V^{-1} V^{-1} m)^{1/2}}$ being coefficients

where $m = (m_1, \dots, m_n)^T$ is a vector constituted of the expected values of the order

The Shapiro-Wilk test was compared to different other tests (Kolmogorov-Smirnov, Anderson-Darling and Lilliefors) via Monte Carlo simulation and has been found to present the best power for a given significance (Razali and Wah, 2011).

Once the test is completed, two different tests are considered depending on the results (normality of the population or not) with the aim of determining if the means of the different samples (in our case control and bexarotene-treated primary cultures) are equal or not and thus underlining the potential effect of the treatment. These tests are Student's t-test and Mann-Whitney's u-test.

Student's t-test

If the tested samples can be considered to come from normally distributed populations (via w-test), Student's t-test is chosen to assess the difference in means. The null hypothesis is that there is no difference in means between the two samples.

Principle

In our case, the t-test is used to determine if the difference in means (if present) between our control and bexarotene-treated groups is significant.

Theory

Here follows the formulas used to carry out the t-test in our case, being an unpaired (independent) t-test

The test statistic is :

$$t = \frac{m_A - m_B}{\sqrt{\frac{S^2}{n_A} + \frac{S^2}{n_B}}}$$

With :

1) $S^2 = \frac{\sum(x - m_A)^2 + \sum(x - m_B)^2}{n_A + n_B - 2}$ being an estimator of the common

variance of the two samples,

2) $df = n_A + n_B - 2$ being the degrees of freedom used in the test,

3) A and B being the two groups to compare (in our case control versus bexarotene treated cultures),

4) m_A and m_B being the means of groups A and B, respectively.

5) n_A and n_B the sizes of groups A and B, respectively.

Once the t-test is carried out, the test statistic is compared to the critical value of Student's t distribution, with a chosen significance level α (in our case $\alpha=0,05$).

If the absolute value ($|t|$) presents a greater value than the critical value found in the t distribution, the difference is considered as significant. The p-value, being the level of

significance, is the probability of an observation being equal or more extreme than the actually observed result.

Mann-Whitney's u-test

Principle

If the tested samples do not come from normally distributed populations (determined via w-test), Mann-Whitney's u-test is chosen to assess the difference in means. The u-test is a non-parametric alternative to the precedently mentioned t-test and is used to compare two samples' means and determine if these are equal or not. The null hypothesis is that it is equally likely that a value selected randomly from a sample will either be smaller or greater than a value selected randomly from a second sample. In order to carry out such a test, four assumptions must be met (as found in <https://statistics.laerd.com>) :

- 1) There needs to be one dependent variable measured at the ordinal or continuous level. In our case, the relative expression level (in fold) is our continuous dependent variable.
- 2) There needs to be one independant variable consisting of independent groups. In our case, this variable is the dichotomous variable control versus bexarotene treated neurons.
- 3) There needs to be an independence of observations. In our case, the independence is the number n of observations (wells in culture plates) in the different independant experiments (primary cultures).
- 4) The u-test can be used when the two variables are not normally distributed

Theory

First, the values are ranked from low to high, then the following formula is applied.

$$U = n_1 n_2 + \frac{n_2(n_2 + 1)}{2} - \sum_{i=n_1+1}^{n_2} R_i$$

With :

U = Mann-Whitney u-test

n_1 = sample size one

n_2 = sample size two

R_i = rank of the sample size

The calculated U is then compared to the critical value (U_{crit}) corresponding to the two sample sizes at a given confidence level (in our case $\alpha=0,05$). If U_{crit} is smaller than the calculated U , the null hypothesis cannot be rejected.

IV) Results

In the following section will be detailed the different data gathered using the methods mentioned previously and statistically processed accordingly.

1) *ABCA1*

ABCA1 expression was assessed using qPCR and western blot analysis according to the fact that bexarotene is an agonist of RXR. As stated before, RXR activation stimulate the expression of cholesterol carrier ABCA1. Therefore, ABCA1 expression is used as positive control, allowing us to confirm or not the effective action of bexarotene on the cell culture.

1.1) PCR results

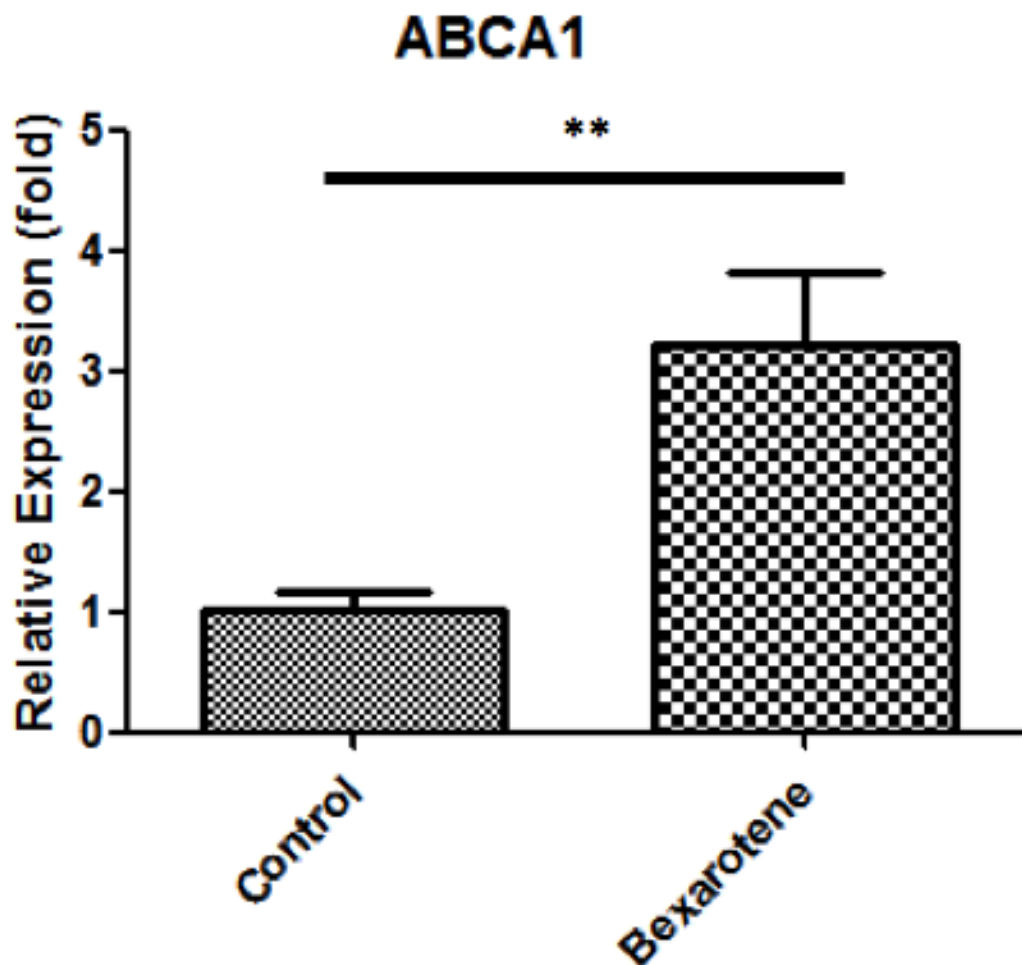


Figure 8 : **Bexarotene increases the expression of ABCA1.** Quantification of the relative expression of ABCA1 protein via qPCR in control primary culture of rat hippocampal neurons (n=9 in 3 independant experiments) and Bexarotene treated primary culture of rat hippocampal neurons (n=9 in 3 independant experiments). The expression difference is stated in folds and the statistical significance (**) has been verified via Student t-test with p-value=0,0025. Normal data distribution verified via Shapiro-Wilk test.

According to the graph here above (Fig. 9), the expression level of the ABCA1 gene assessed through qPCR is higher in the presence of bexarotene. The difference seems to be of the three-fold order and is found to be, accordingly, significant via t-test (p-value = 0,0025). The standard deviation in the bexarotene-treated group is higher than in the control group

1.2) Western blot results

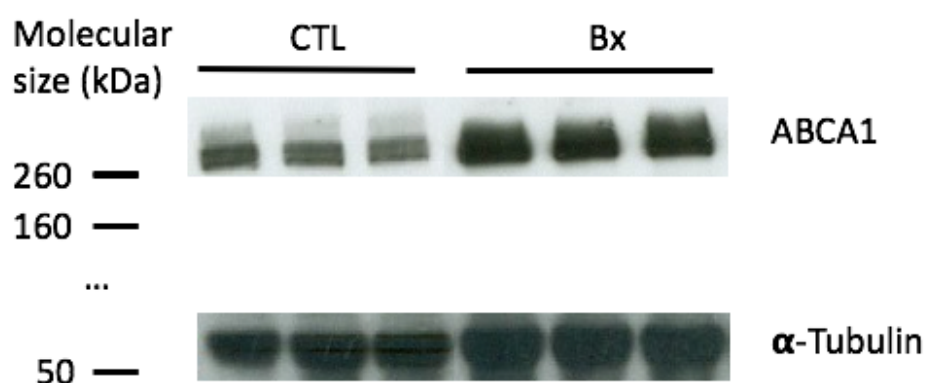


Figure 9 : Bexarotene increases the expression of ABCA1. Western blot analysis of the relative expression of ABCA1 protein and alpha-tubulin for standardization in lysate (20µg) of control (CTL) primary culture of rat hippocampal neurons and Bexarotene (Bx) treated primary culture of rat hippocampal neurons.

We can see in figure 10 that the expression of ABCA1 seems to increase in presence of the RXR agonist as suggested in the PCR analysis. Though the background noise present on the blot seems to be high, it did not affect the significance of the expression levels difference in means (Fig. 11).

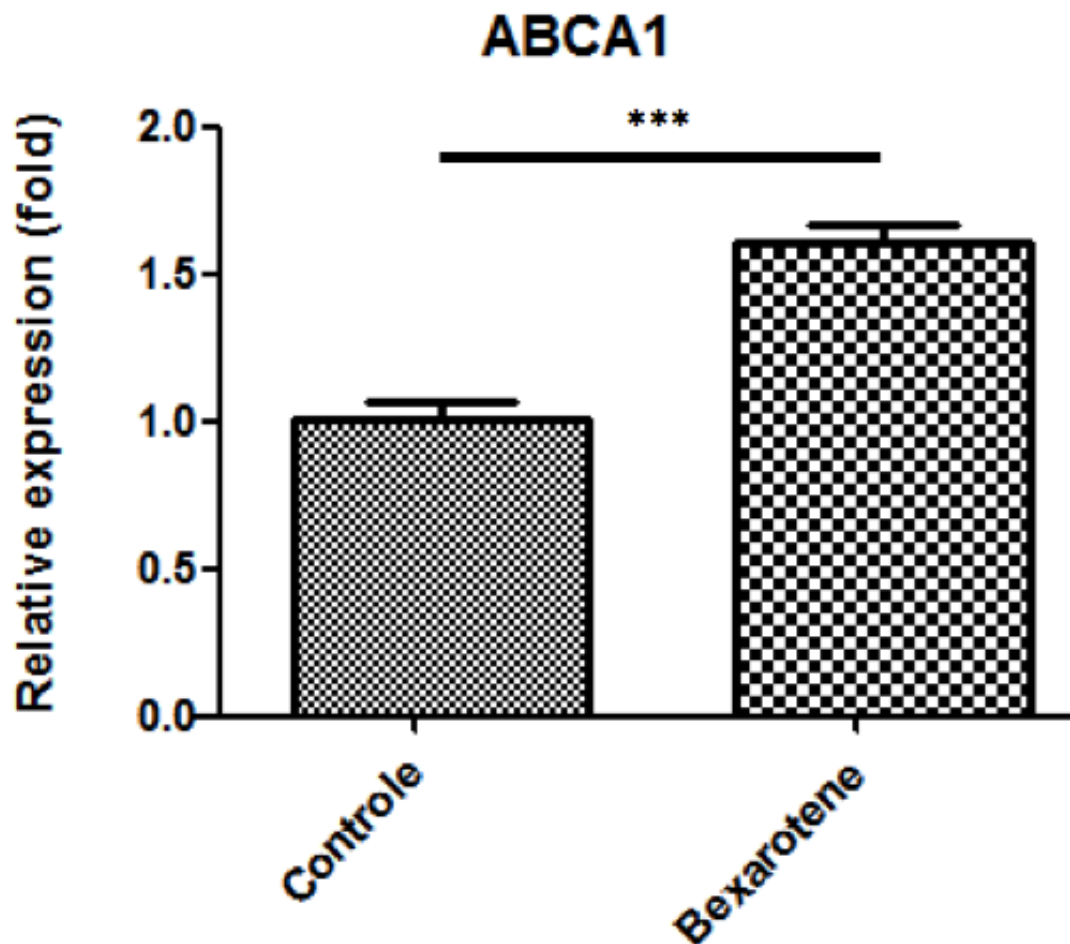


*Figure 10 : Bexarotene increases the expression of ABCA1. Quantification of the relative expression of ABCA1 protein via western blot analysis of lysate (20µg) of control primary culture of rat hippocampal neurons (n=9 in 3 independant experiments) and Bexarotene treated primary culture of rat hippocampal neurons (n=9 in 3 independant experiments). The expression difference is stated in folds and the statistical significance (***) has been verified via Student t-test with p-value < 0,0001). Normal data distribution verified via Shapiro-Wilk test.*

The statistical analysis of the relative expression levels quantification obtained via western blot shows that the presence of bexarotene increases the expression of ABCA1. Also, as we can see, the increase in expression level seem to be of around 1,5-fold, while in the PCR analysis the increase is twice greater. This discrepancy can be explained first by the distribution of the data in the PCR analysis (as stated before) but also by the fact that the qPCR analysis measures the number of gene copies in mRNA while western blot analysis relates to the end-product, being the protein. While being parts of the same general process of gene expression, post-translational modifications occur along with other mechanisms, making the assumption of a direct causative link between mRNA and protein concentrations misguided (Greenbaum et al., 2003).

2) Ionotropic glutamate receptors

2.1) AMPAR

As previously mentioned, AMPA receptors are the main responsible receptors in fast excitatory neurotransmissions in the mammalian brain. Following that logic, two different subunits (GluA1 and GluA2) of the AMPA receptors family were investigated in the presence or absence of bexarotene, via PCR and western blot analyses.

2.1.1) PCR results

GluA1

Here follow the results of the PCR analysis of the first AMPAr subunit, GluA1.

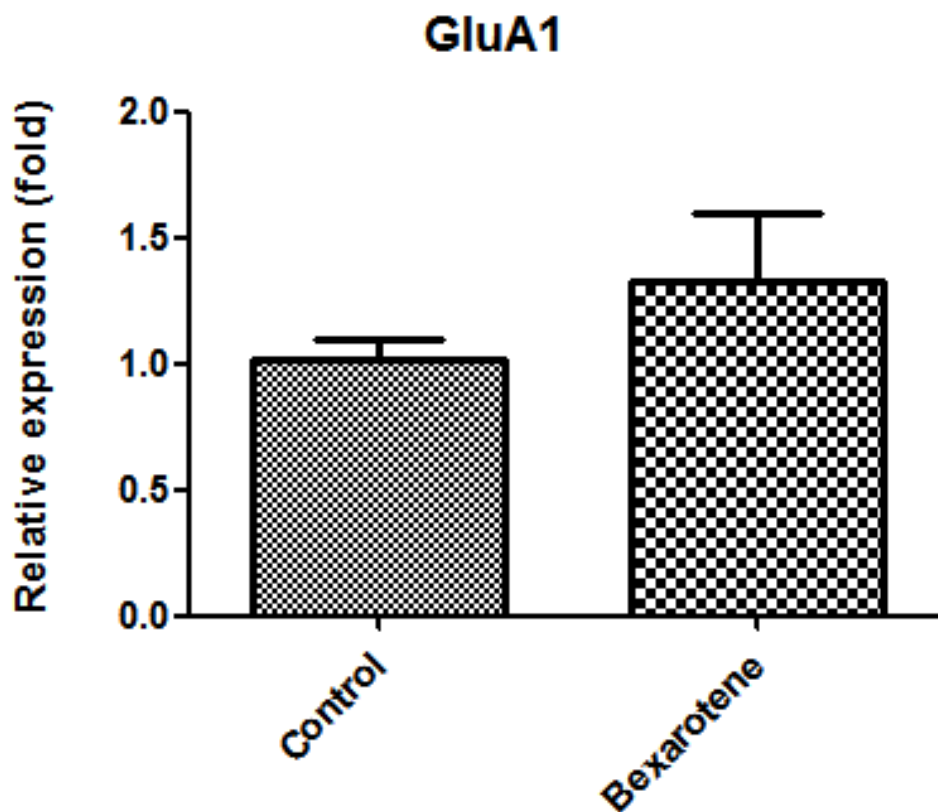


Figure 11 : Quantification of the relative expression of GluA1 subunit of AMPA receptors via qPCR in control primary culture of rat hippocampal neurons (n=10 in 3 independent experiments) and Bexarotene treated primary culture of rat hippocampal neurons (n=6 in 2 independent experiments). The expression difference is stated in folds. Normal data distribution could not be verified via Shapiro-Wilk test in control nor in Bexarotene treated (n too small) and thus Mann-Whitney test was applied with p-value = 0,1286.

In the mRNA concentrations analysed via PCR, GluA1 expression seems to be greater in the presence of the RXR agonist (Fig. 12), but as the error bar suggests, no significant

difference in means can be drawn. We can also notice that the error factor is significantly greater in the data of bexarotene-treated neurons.

2.1.2) Western blot results

GluA1

An example of western blot result reflecting the main pattern observed is showed as figure 13. For a comparable and consistent background noise, the expression levels of GluA1 in both conditions seem similar.

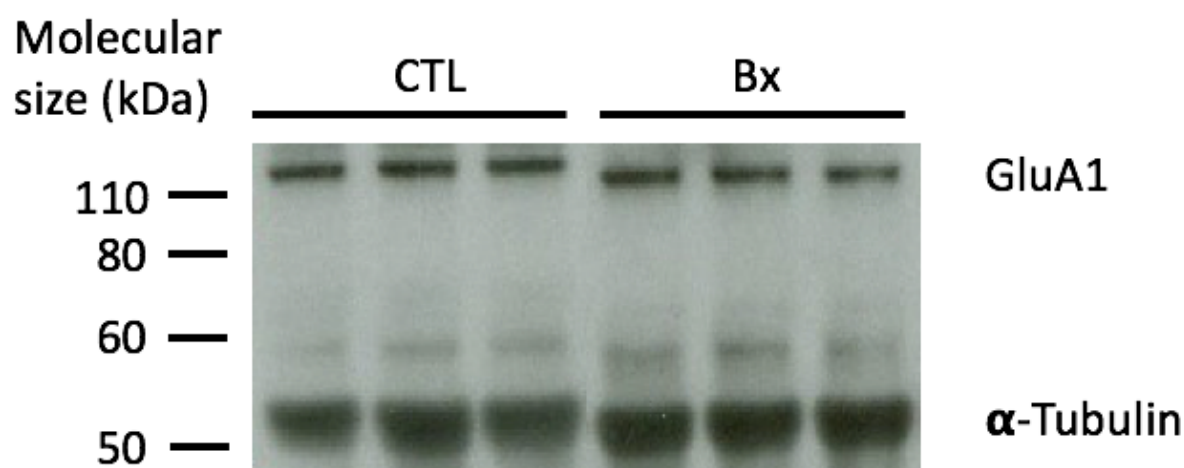


Figure 12 : Western blot analysis of the relative expression (in fold) of GluA1 subunit of AMPA receptors and alpha-tubulin for standardization in lysate (20 μ g) of control (CTL) primary culture of rat hippocampal neurons and Bexarotene (Bx) treated primary culture of rat hippocampal neurons.

The quantification of GluA1 expression blots yielded the following results (Fig. 14). As we can see, GluA1 expression remains unchanged upon bexarotene treatment. Interestingly, there is a discrepancy between PCR and western blot results, as the former suggests an upregulation and the latter a slight downregulation. The great error parameter in the PCR analysis is to be held responsible, as the western blot analysis yielded consistent results throughout the study.

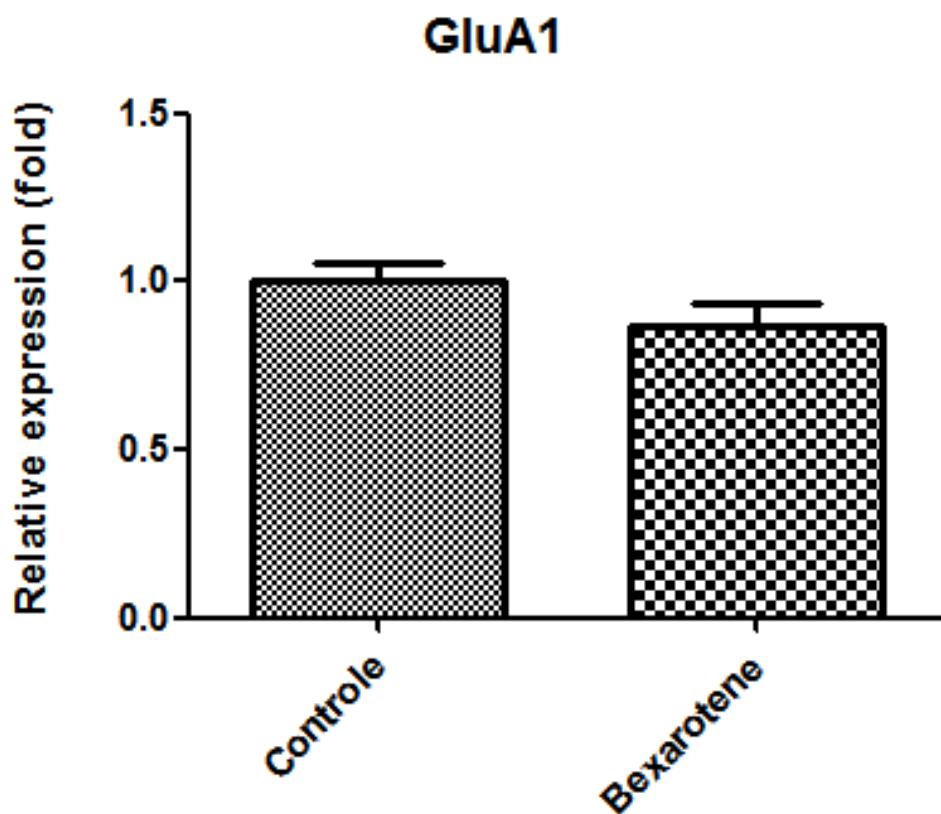


Figure 13 : Quantification of the relative expression (in fold) of GluA1 subunit of AMPA receptors via western blot analysis

GluA2

The western blot analysis of GluA2 yielded the following results.

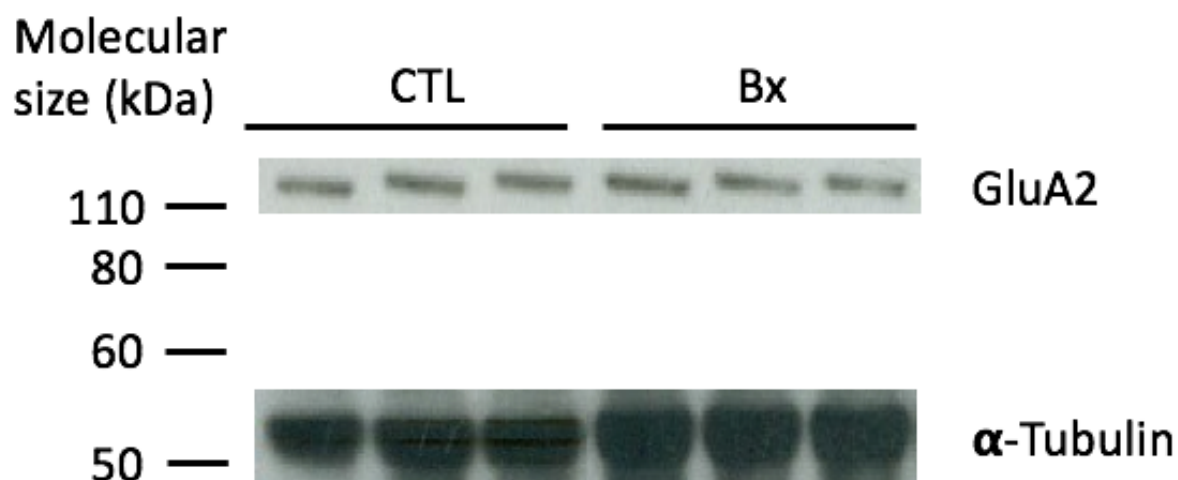


Figure 14 : Western blot analysis of the relative expression (in fold) of GluA2 subunit of AMPA receptors and alpha-tubulin for standardization in lysate (20 μ g) of control (CTL) primary culture of rat hippocampal neurons and Bexarotene (Bx) treated primary culture of rat hippocampal neurons.

We can observe in figure 15 here above that the GluA2 concentration in western blot analysis does not seem to be affected by the presence of bexarotene.

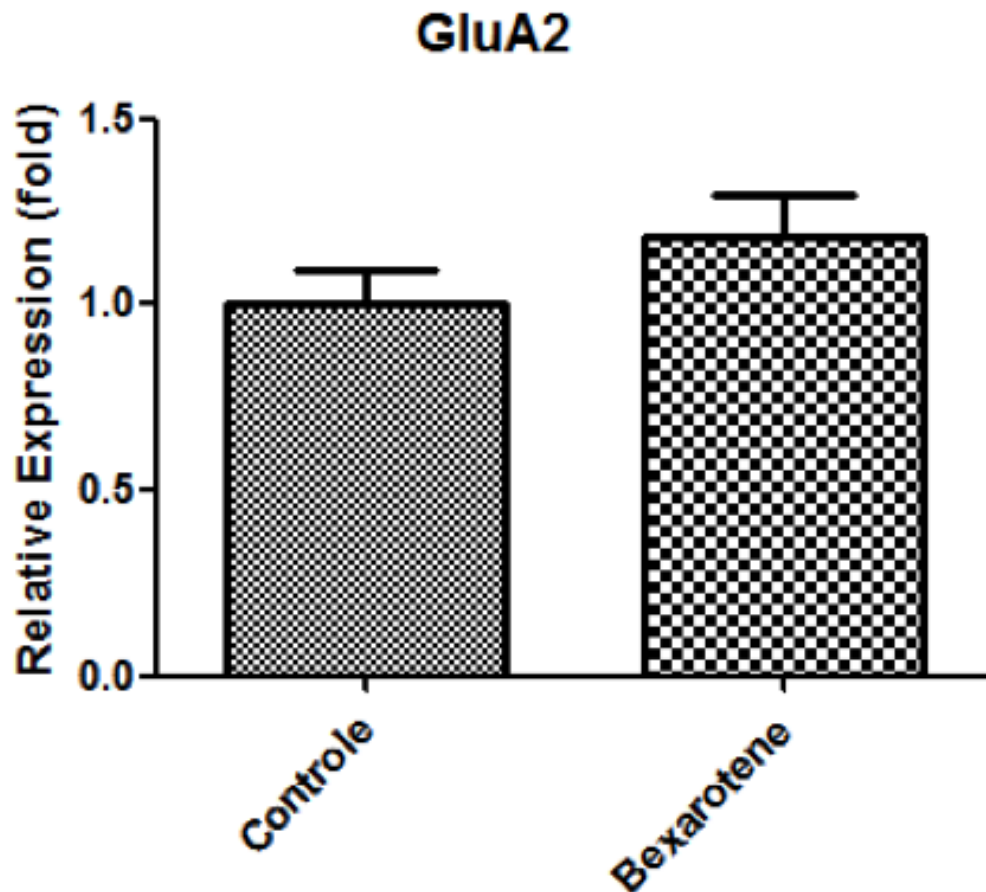


Figure 15 : Quantification of the relative expression of GluA2 subunit of AMPA receptors via western blot analysis of lysate (20 μ g) of control primary culture of rat hippocampal neurons (n=11 in 3 independant experiments) and Bexarotene treated primary culture of rat hippocampal neurons (n=11 in 3 independant experiments). The expression difference is stated in folds and no statistical difference could be verified via Student t-test (p-value = 0,2270). Normal data distribution verified via Shapiro-Wilk test.

Figure 16 here above shows a slight increase in expression of GluA2 subunit in western blot analysis. However, Student's t-test yielded a p-value of 0,2270 > 0,05, meaning that this difference cannot be considered as significant.

2.2) NMDAR

NMDA receptors play a key role in the development of synaptic plasticity. Accordingly, these receptors were investigated via PCR and western blot analyses of two different subunits, GluN2A and GluN2B.

2.2.1) PCR results

GluN2A

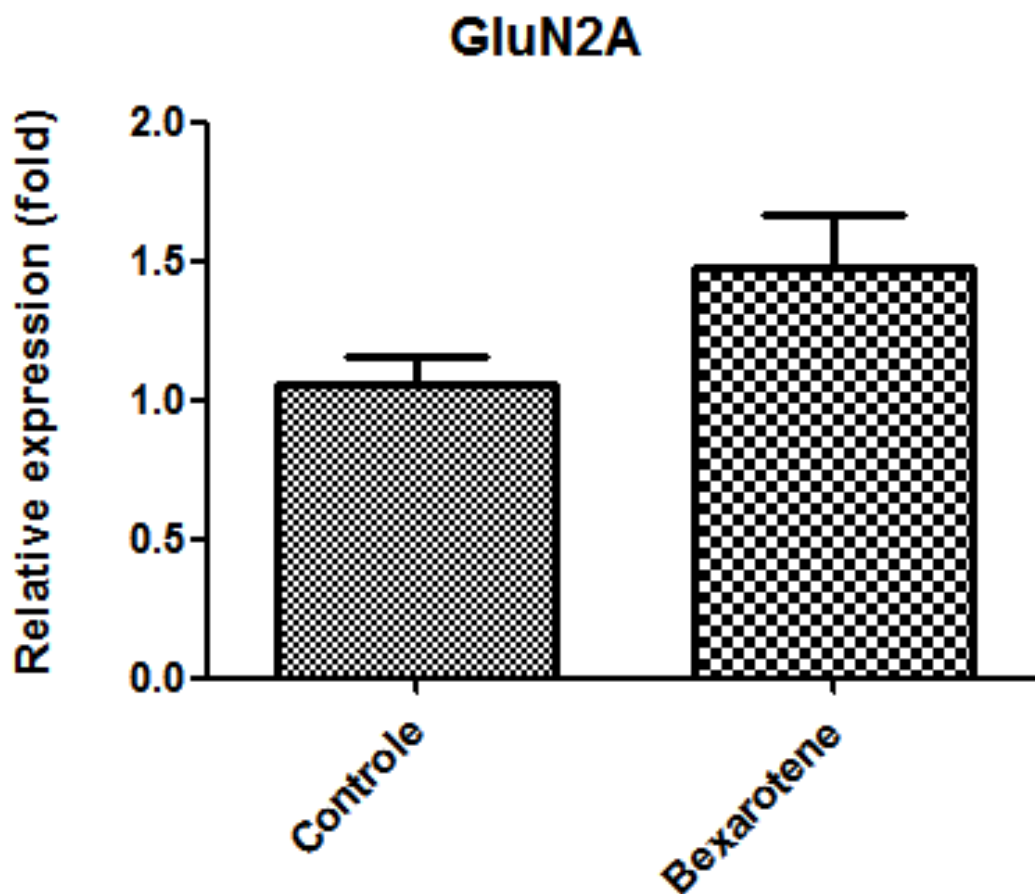


Figure 16 : Quantification of the relative expression (in fold) of GluN2A subunit of NMDA receptors via qPCR in control primary culture of rat hippocampal neurons and Bexarotene treated primary culture of rat hippocampal neurons. The expression difference is stated in folds and no statistically significant difference could be verified via Student t-test (p -value=0,0839). Normal data distribution verified via Shapiro-Wilk test.

The PCR analysis of GluN2A (Fig. 17) shows that in the presence of bexarotene, there seems to be an upregulation. However, the p -value obtained is greater than $\alpha=0,05$, meaning that this difference is not significant.

GluN2B

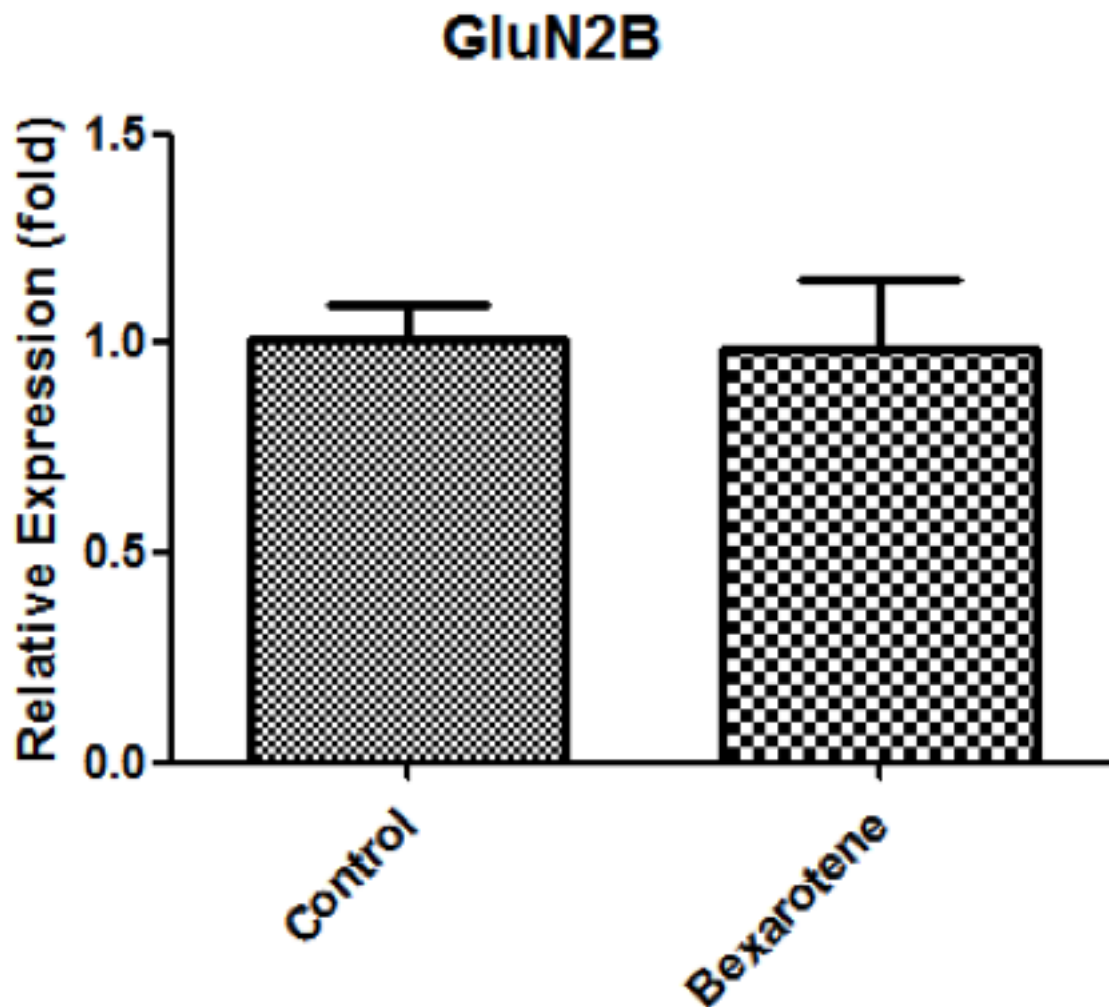


Figure 17 : Quantification of the relative expression (in fold) of GluN2B subunit of NMDA receptors via qPCR in control primary culture of rat hippocampal neurons (n=10 in 3 independant experiments) and Bexarotene treated primary culture of rat hippocampal neurons (n=8 in 3 independant experiments). The expression difference is stated in folds and no statistically significant difference could be verified via Student t-test (p-value=0,9006). Normal data distribution verified via Shapiro-Wilk test.

The presence of bexarotene does not seem to affect (Fig. 18) the expression of the other subunit studied, GluN2B.

2.2.2) Western blot results

GluN2A

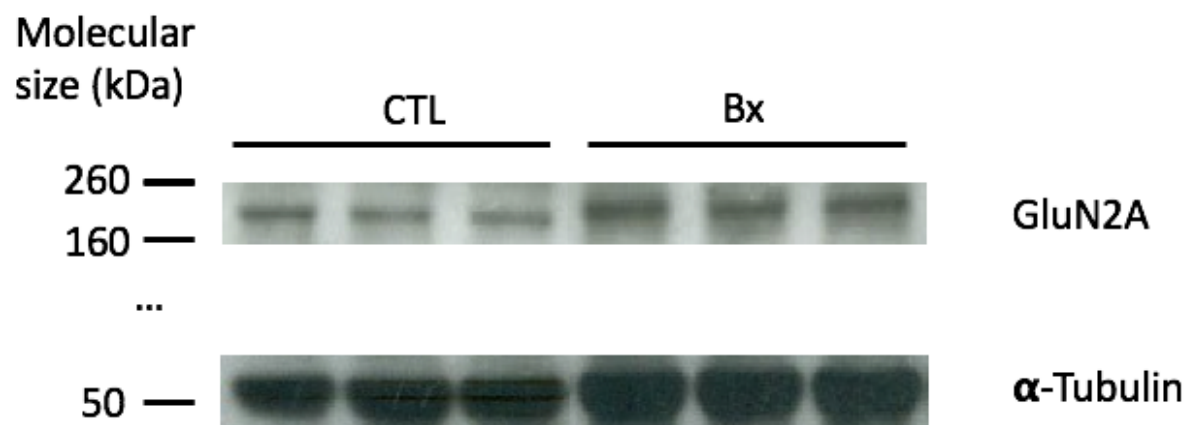


Figure 18 : Bexarotene increases the expression of GluN2A. Western blot analysis of the relative expression of GluN2A subunit of NMDA receptors and alpha-tubulin for standardization in lysate (20µg) of control (CTL) primary culture of rat hippocampal neurons and Bexarotene (Bx) treated primary culture of rat hippocampal neurons.

The western blot analysis of the GluN2A subunit revealed an upregulation in presence of bexarotene, as seen in figure 19.

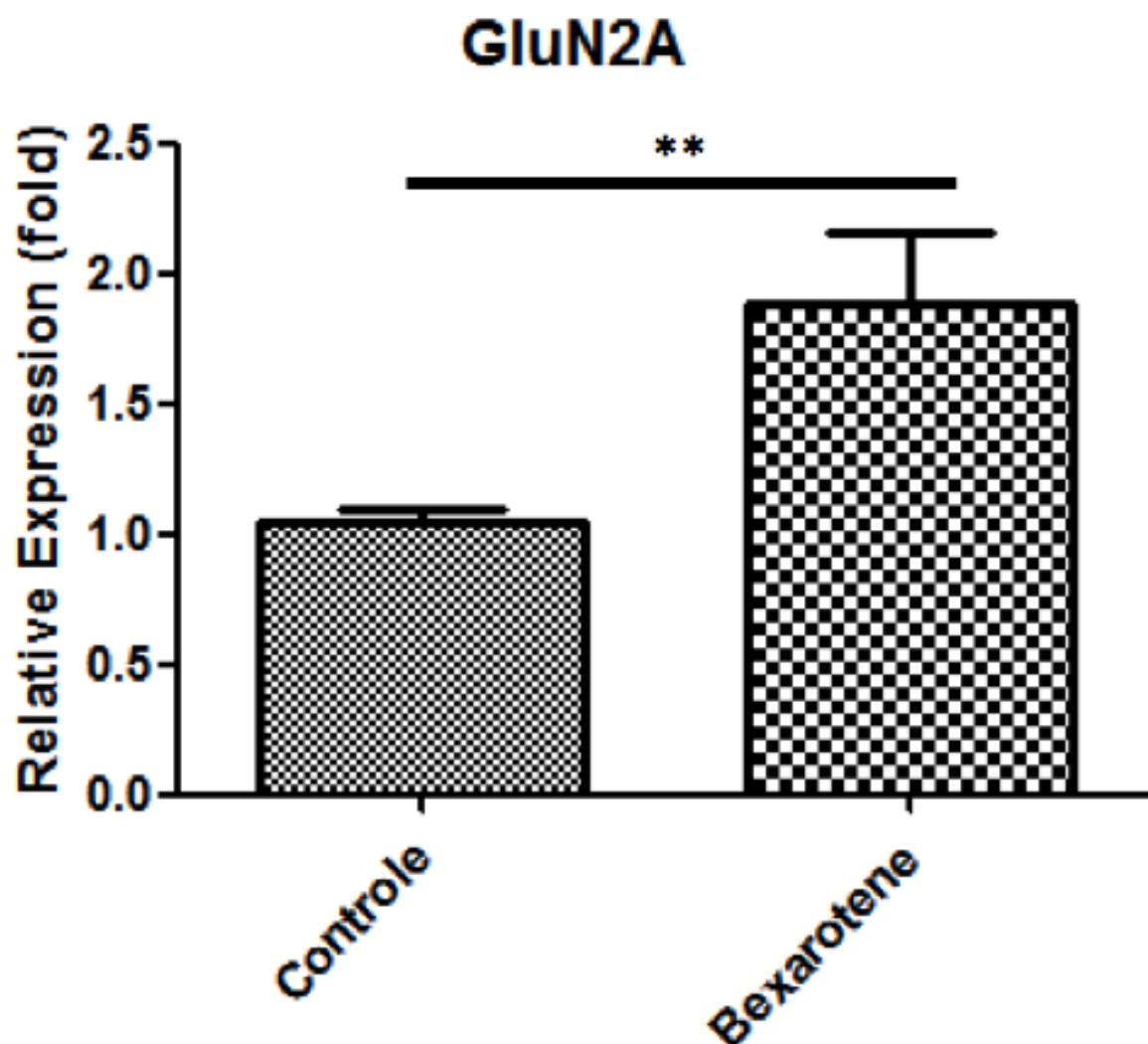


Figure 19 : **Bexarotene increases the expression of GluN2A.** Quantification of the relative expression of GluN2A subunit of NMDA receptors via western blot analysis of lysate (20µg) of control primary culture of rat hippocampal neurons (n=10 in 3 independant experiments) and Bexarotene treated primary culture of rat hippocampal neurons (n=10 in 3 independant experiments). The expression difference is stated in folds and the statistical significance (**) has been verified via Student t-test with $p\text{-value} = 0,0083$). Normal data distribution verified via Shapiro-Wilk test.

The increase of GluN2A subunit expression is confirmed by the quantification (Fig. 20). In the presence of the RXR agonist, the NMDAr subunit expression increase seems to be of a two-fold order, with a $p\text{-value}$ of 0,0083.

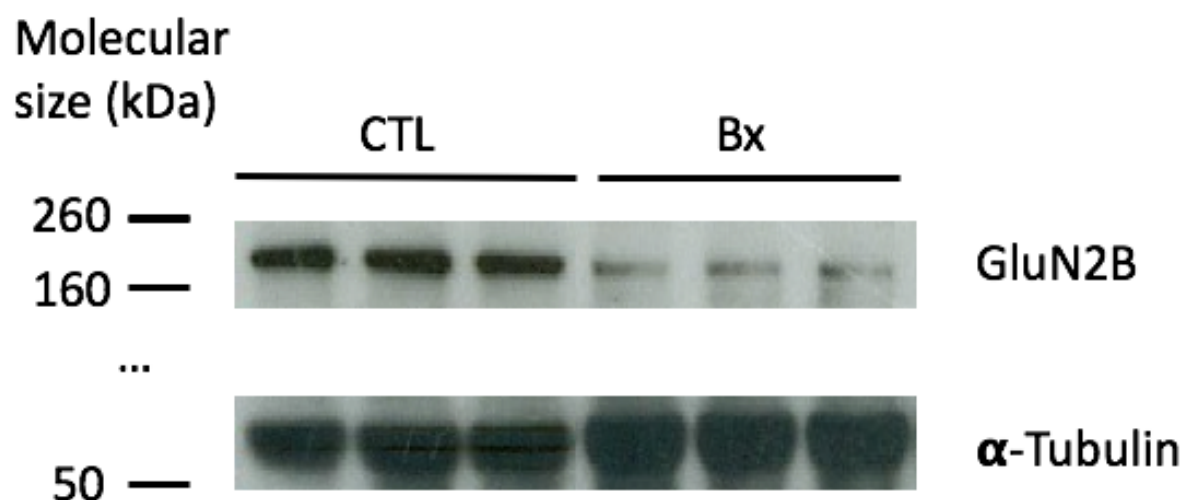


Figure 20 : Western blot analysis of the relative expression of GluN2B subunit of NMDA receptors and alpha-tubulin for standardization in lysate (20 μ g) of control (CTL) primary culture of rat hippocampal neurons and Bexarotene (Bx) treated primary culture of rat hippocampal neurons.

As we can see in figure 21, the presence of bexarotene presents an effect on the expression of GluN2B. The quantification of these results (Fig. 22) confirms the significant (p-value = 0,0023) difference in means between the two conditions. Indeed, the presence of the RXR agonist appears to diminish the expression of the GluN2B subunit.

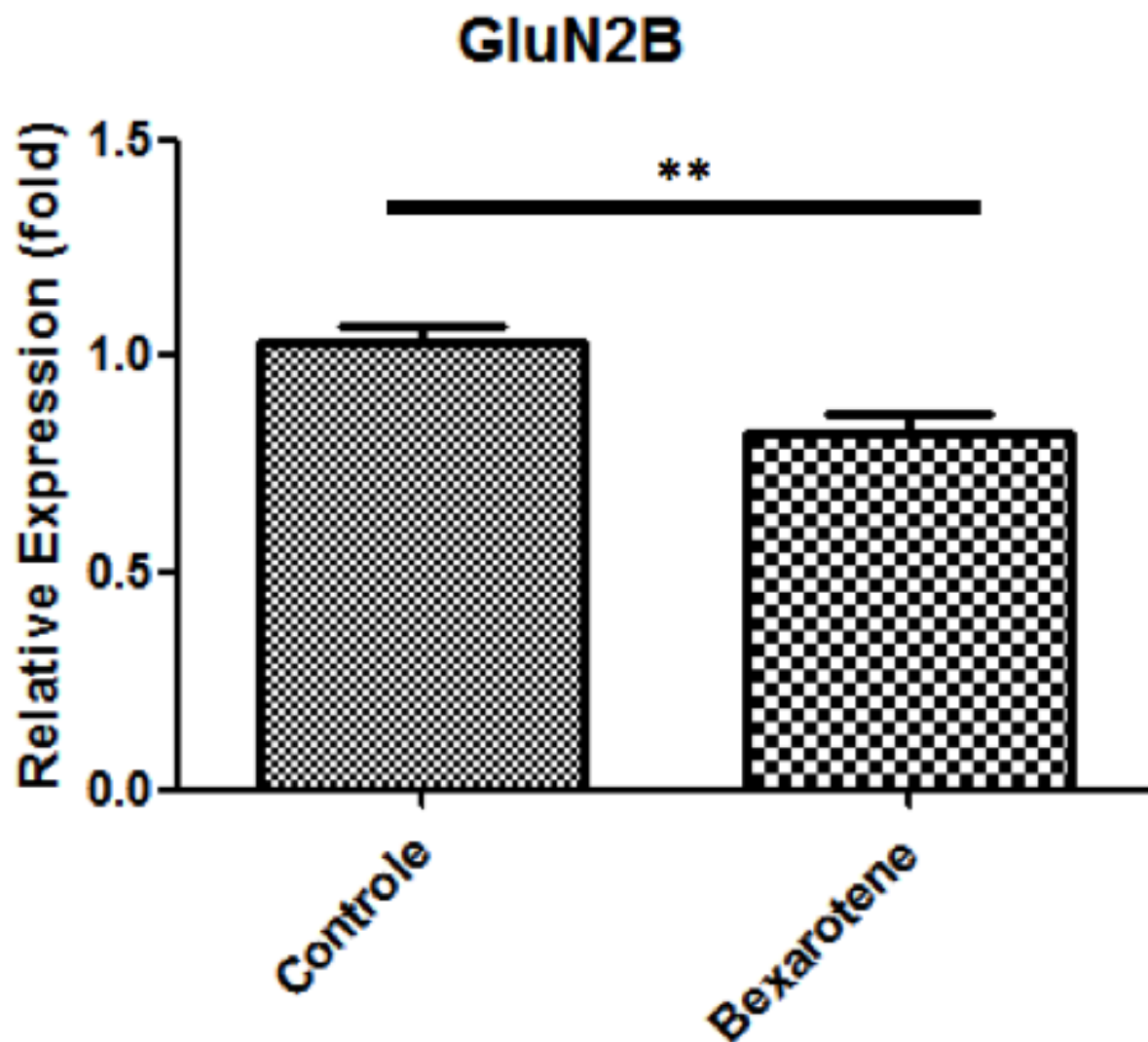


Figure 21 : Quantification of the relative expression (in fold) of GluN2B subunit of NMDA receptors via western blot analysis of lysate (20 μ g) of control primary culture of rat hippocampal neurons (n=10 in 3 independant experiments) and Bexarotene treated primary culture of rat hippocampal neurons (n=10 in 3 independant experiments). The expression difference is stated in folds and the statistical significance (**) has been verified via Student t-test with p-value = 0,0023). Normal data distribution verified via Shapiro-Wilk test.

3) Synaptophysin

The effect of bexarotene on the presynaptic glycoprotein synaptophysin expression was tested due to the fact that this expression has been shown to decrease in AD patients, specifically in temporal as well as frontal cortex and hippocampus, leading to synapse disruption. As mentioned before, synaptophysin plays a role in the regulation of activity-dependent synaptic formation by acting on the fusion between vesicles and the presynaptic membrane. The relative expression depending on the presence of bexarotene was assessed via western blot analysis on primary cultures of rat embryonic hippocampal neurons.

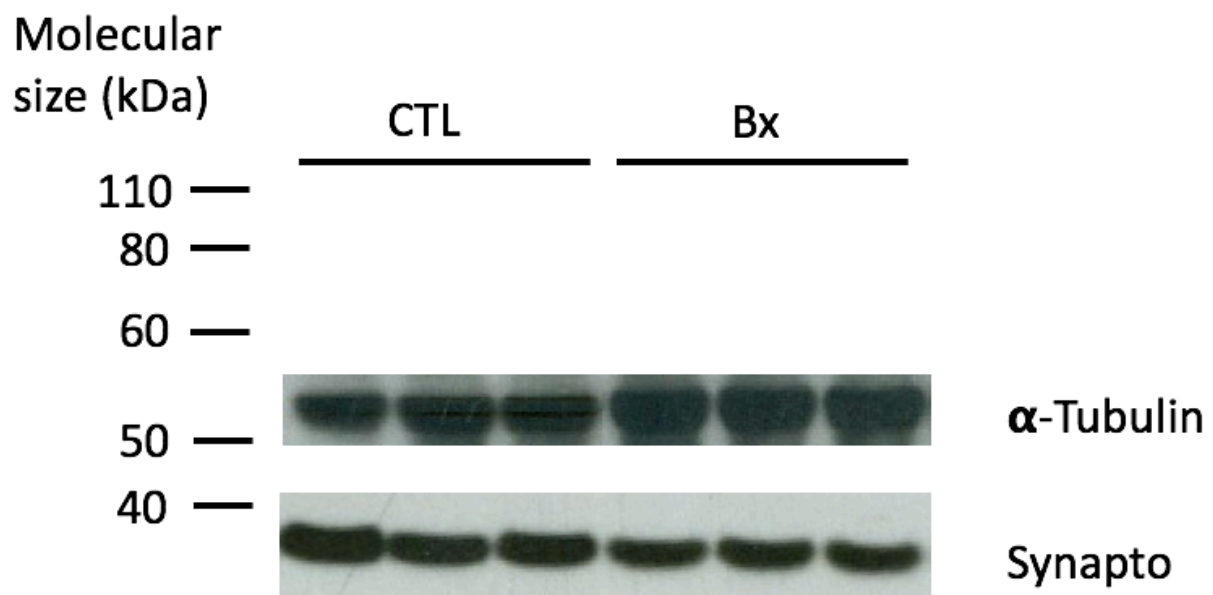


Figure 22 : Western blot analysis of the relative expression of Synaptophysin (Synapto) and alpha-tubulin for standardization in lysate (20 μ g) of control (CTL) primary culture of rat hippocampal neurons and Bexarotene (Bx) treated primary culture of rat hippocampal neurons.

The relative expression of synaptophysin does not seem to be affected by the presence of bexarotene (Fig. 22).

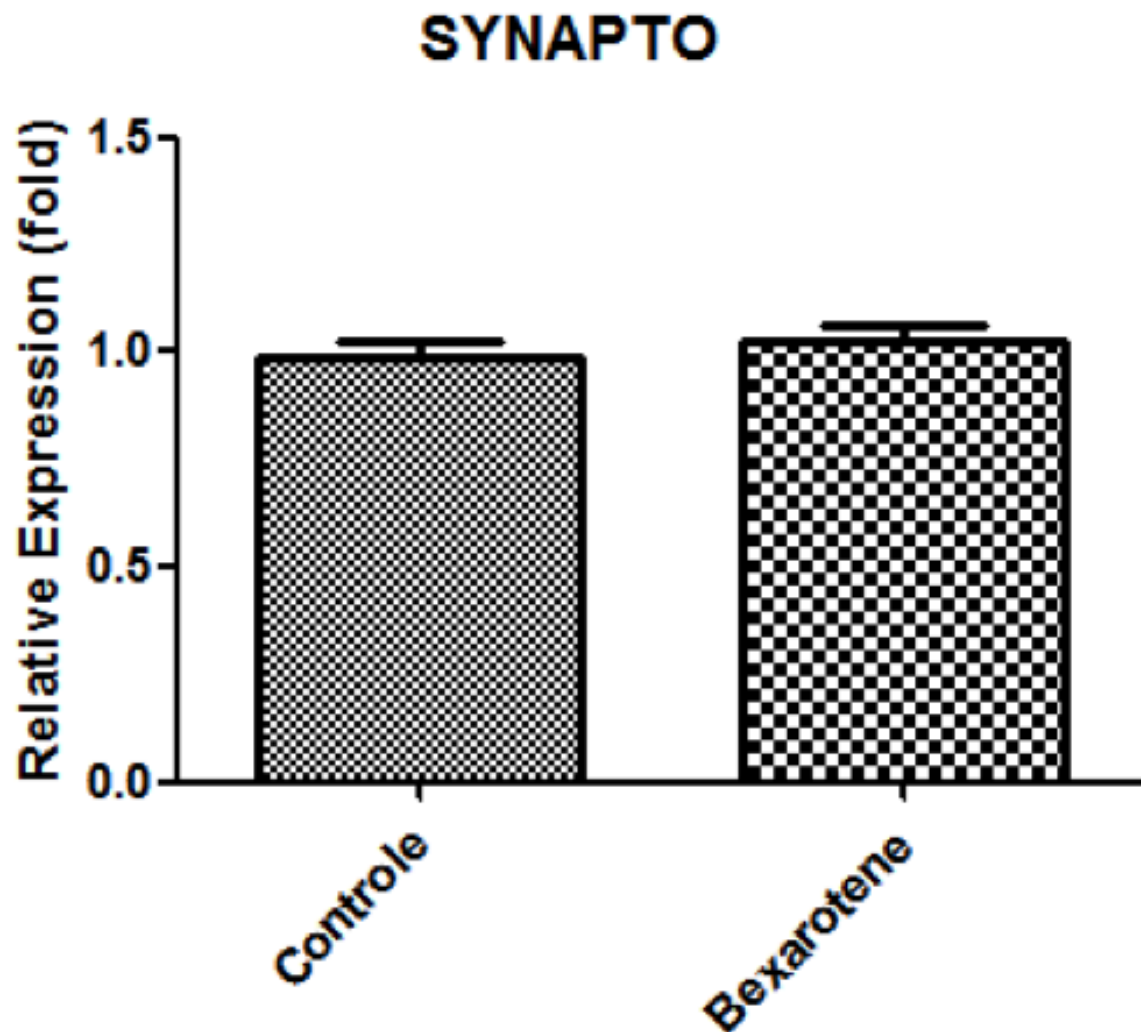


Figure 23 : Quantification of the relative expression of synaptophysin protein (Synapto) via western blot analysis of lysate (20 μ g) of control primary culture of rat hippocampal neurons (n=9 in 3 independant experiments) and Bexarotene treated primary culture of rat hippocampal neurons (n=9 in 3 independant experiments). The expression difference is stated in folds and no statistical significance could be verified via Student t-test (p-value = 0,3292). Normal data distribution verified via Shapiro-Wilk test.

The quantification of the blot (Fig. 23) confirms that the expression of synaptophysin is, in our case, not affected by the RXR agonist.

4) PSD95

As explained earlier, PSD95 is involved in the formation of clusters containing different receptors such as NMDAR and AMPAR at the post-synaptic density of neurons. Moreover, it has been stated that both synaptophysin and PSD95 expression levels in the hippocampus are decreased in AD patients. Accordingly, a western blot analysis has been carried out to determine the potential effect of bexarotene on the expression of PSD95 in wild type hippocampal neurons.

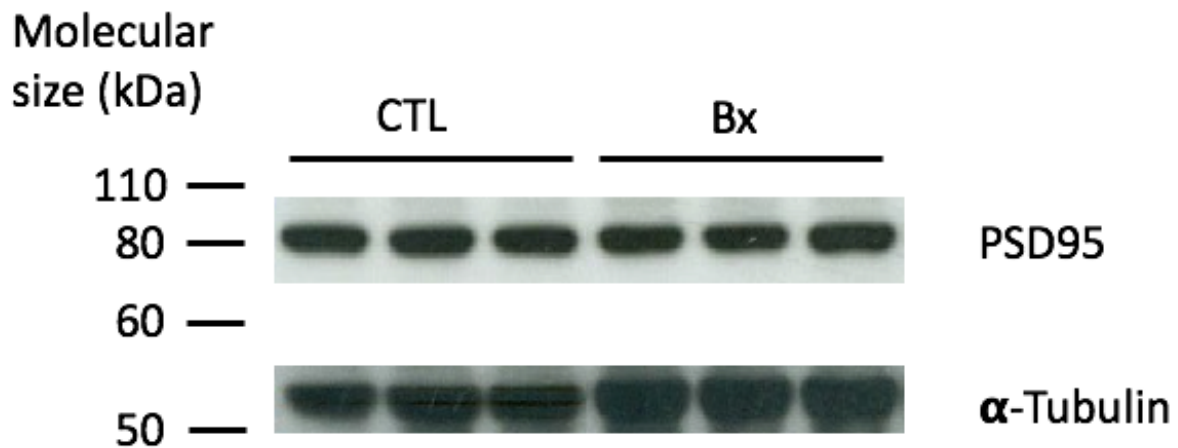


Figure 24 : Western blot analysis of the relative expression of PSD95 protein and alpha-tubulin for standardization in lysate (20µg) of control (CTL) primary culture of rat hippocampal neurons and Bexarotene (Bx) treated primary culture of rat hippocampal neurons.

In figure 24, we can see that the presence or absence of bexarotene does not seem to impact the expression level of the post-synaptic protein PSD95.

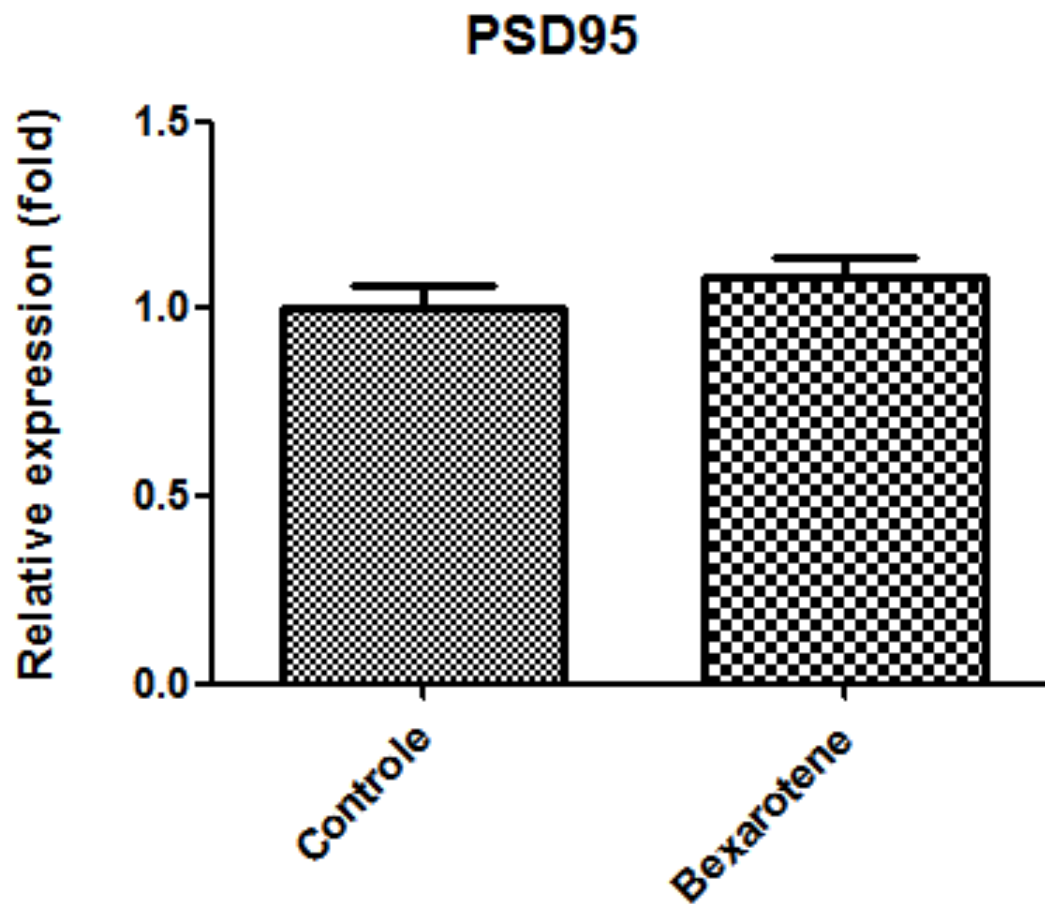


Figure 25 : Quantification of the relative expression (in fold) of PSD95 protein via western blot analysis of lysate (20 μ g) of control primary culture of rat hippocampal neurons (n=9 in 3 independant experiments) and Bexarotene treated primary culture of rat hippocampal neurons (n=9 in 3 independant experiments). The expression difference is stated in folds and no statistical significance could be verified via Student t-test (p-value = 0,3292). Normal data distribution verified via Shapiro-Wilk test.

The quantification is in accordance with the previous statement, being that no noticeable effect of bexarotene on PSD95 expression can be observed.

V) Discussion

In this section will first be discussed both the relevance of the model used and the different results (and their significance) obtained throughout the study, and secondly the potential perspectives that could be considered according to the results.

1) Discussion of the model

The use of primary cultures of rat embryonic hippocampal neurons finds its relevance in different aspects. Notably the fact that the hippocampus is generally viewed as a key location for the establishment of different memory-related mechanisms and, moreover, the confirmed presence of proteins associated with RXR (LXR, PPAR), being the target of bexarotene, in the hippocampus. The interest of a primary culture of neurons is, in our case, the ability to reform functional synaptic networks. However, such model holds certain drawbacks. Among these is the susceptibility of primary cultures to contamination by external agents and also the fact that such a culture requires a relatively long time (about two weeks) to develop and be ready to work with, meaning that a contamination occurring around the last days of culture would substantially delay one's opportunity to carry out experiments. Moreover, knowing that the hippocampus of a rat embryo is quite small, the number of neurons that can be harvested is significantly smaller than the number of cortical neurons that could be gathered from a given rat embryo. This leads inevitably to a smaller number of observations per experiment, impacting in turn the potential statistical significance of said observations.

2) Discussion of the results

In the following will be recapitulated and reviewed the different results obtained, as well as critics regarding their accordance or not to previous works.

2.1) ABCA1 expression increases in presence of bexarotene

The results obtained either via quantitative polymerase chain reaction or via western blot analysis concur with previous studies stating that bexarotene treatment causes significant upregulation of ABCA1 and ApoE proteins, both involved in the reverse transport of cholesterol in the central nervous system.

This upregulation of bexarotene's target gene therefore lets us presume that the treatment applied to the primary cultures has indeed been effective.

2.2) The response of AMPA receptors subunits GluA1 and GluA2 to bexarotene is not well defined

GluA1

The relative expression of the GluA1 subunit of AMPA receptors in presence of bexarotene yielded inconsistent results. While the qPCR analysis suggested an upregulation (though not statistically significant) of GluA1 in the treated neurons, the protein assay showed no apparent effect except maybe a slight downregulation. The standard error is quite important in the case of qPCR analysis, denying the possibility to distinguish a pattern or draw any relevant conclusion.

GluA2

The treatment with bexarotene showed a slight upregulation of GluA2 but failed to be statistically significant. However, if this trend was to be confirmed in following studies, a potential effect of this upregulation would be the shift in proportion of GluA2-containing versus GluA2-lacking AMPA receptors. This increase in GluA2-containing AMPA receptors would lead to, for example, a decrease in channel conductance as well as calcium ions permeability. This, in turn would substantially affect synaptic activity. As the calcium permeability decreases, the neurons become less likely to reach the threshold required to fire an action potential, therefore contributing to synaptic inhibition. Interestingly, some studies (Busche and Konnerth, 2015 ; Busche et al., 2018) pointed the fact that neurons located nearby amyloid plaques show signs of hyperactivity, which could be due to a

decrease in synaptic inhibition. The increase in GluA2-containing AMPA receptors could then be favourable for the reinstatement of normal neuronal activity around the amyloid plaques.

2.3) Bexarotene could impact the subunit ratio of NMDA receptors

According to the results, the RXR agonist appears to affect differently the two NMDA receptor subunits studied.

GluN2A

In this study, bexarotene upregulates GluN2A, as shown by qPCR and confirmed via western blot analyses (though only the protein assay led to a statistically significant difference in expression). This upregulation appears to be of 1,5-2 fold order. This effect could be beneficial, as some studies have shown that GluN2A activation appears to be neurotrophic (Liu et al., 2007 ; Lai et al., 2011). However, it has to be pointed that these results correspond to studies focused on stroke-related damages and were carried out on cortical neurons.

GluN2B

Opposingly to GluN2A results, bexarotene downregulates GluN2B (western blot results) or does not affect the expression (qPCR). This downregulation, however, is quite modest.

GluN2A/GluN2B ratio

As mentioned before by Cull-Candy and his team in 2001, the GluN2A/GluN2B ratio could play a role in the difference of kinetics of the NMDA receptors. GluN2B being less expressed, it would make place for more GluN2A-containing NMDARs exhibiting faster kinetics which in turn would mean a faster return to baseline excitation level. This shift in subunit ratio could be responsible for a decrease in the excited-state time of NMDA receptors. As this duration diminishes, the amount of calcium ions passing through the channel diminishes as well, meaning a decrease in intracellular calcium ions concentration. While being primordial for healthy neurons in order to generate action potentials, high

intracellular calcium ions concentration can lead to the generation of reactive oxygen species (ROS) leading to neurons suffering from excitotoxicity ; simply put, as in the study led by Zhou and Sheng in 2013, « NMDARs can cause excitotoxicity », but their synaptic activity is still vital for neurons.

It has to be stated that the subsynaptic location of the NMDAR has an incidence on the potential excitotoxicity (Zhou and Sheng, 2013). Their consensus is that extrasynaptic NMDARs are more likely to trigger cell death mechanisms than synaptic NMDARs, and that GluN2B-containing NMDARs seem to be preferentially distributed in extrasynaptic regions.

2.4) PSD 95 and Synaptophysin

The gathered results concerning PSD95 and Synaptophysin are similar, in the way that the presence of bexarotene does not appear to have any effect on the expression of either protein. In this case, the potential beneficial effects of bexarotene do not include PSD95 and Synaptophysin in the target proteins.

VI) Conclusion and perspectives

While some results were expected and in accordance with what was previously shown in anterior studies, some others came out quite unexpected. The upregulation of ABCA1 would assert that the bexarotene treatment was effective ; But the erratic results concerning the effects on GluA1 are puzzling, as previous works suggested that the mode of action of the RXR agonist involved the upregulation of the AMPAR subunit. More experiments, though expensive and time-consuming, concerning the effects of bexarotene on AMPAR in primary cultures of hippocampal neurons could resolve this issue.

The apparent absence of effects on the expression of the synaptic proteins PSD95 and Synaptophysin does not offer a promising path to investigate the molecular action of bexarotene.

From a general point of view, what emerges from the gathered results is the effect of the treatment on the two studied NMDAR subunits, GluN2A and GluN2B. While the suggested involvement of the upregulation of the former and downregulation of the latter are only hypotheses, further study could be of interest in order to confirm or invalidate the potential role of NMDAR subunit composition and subsynaptic location in hippocampal neurons. Such studies could benefit from the use of murine models of AD for the different analyses rather than wild-type animals, as the the pathogenesis mechanisms could alter the observed effects. Bexarotene effects on peroxysome proliferator-activated receptors and subsequent action on the heterodimers formed with RXR are an interesting path to investigate. Another example could be the use of immunocytochemistry to determine the subcellular locations of AMPAR and NMDAR and the inherent consequences.

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Influence of retinoid X receptors activation on the expression of ionotropic glutamate receptors

Presented by Paul-Emile de Sauvage

Summary

Alzheimer's disease (AD) is a neurodegenerative disorder first described in 1906. The disease is characterized by the progressive and irreversible loss of neurons in the central nervous system (CNS) leading to loss of cognitive functions, including memory. The current paradigm of AD includes two main lesions, being the intracellular neurofibrillary tangles and the extracellular amyloid β ($A\beta$) plaques. Numerous therapies aimed at reducing the amyloid deposits through various treatments were conducted. It was shown that while the cellular symptoms were indeed impacted, the cognitive functions of the patients were not well reestablished.

In 2015, professor Octave with N. Pierrot (phD) and their team shed light on the case of a patient suffering from AD and was given a treatment commonly used against cutaneous T cell lymphoma, with the consequences of improved cognitive functions. This treatment was bexarotene, an agonist of retinoid X receptors acting on lipid homeostasis. The purpose of this work was to try to understand the underlying mechanisms behind the beneficial effects of bexarotene on cognitive functions. This was performed through the analysis of the expression of various genes proven to be essential in the establishment of memory processes, including genes coding for subunits of receptors involved in synaptic plasticity (strengthening of neuronal junctions). In addition, the expression of a cholesterol transporter was also assayed as its role in bexarotene action has been proven.

Primary cultures of hippocampal neurons of rat embryos were treated with bexarotene for 24 hours before harvesting, allowing the comparison of relative gene expression against non-treated neurons (control group). Reverse transcription and quantitative polymerase chain reaction were carried out to measure the messenger RNA and western blotting was used to measure the relative expression of proteins. The results gathered throughout this study suggest the modification of subunit ratio of NMDA (N-methyl-D-aspartate) synaptic receptors as a potential part of bexarotene mode of action. Some effects on AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors were also observed but no consistent explanation could be drawn.

