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Identification of novel regulatory mechanisms to control cholesterol homeostasis: a focus on brain cells

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Identification of novel regulatory mechanisms to control cholesterol homeostasis: a focus on brain cells

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ABSTRACT

Cholesterol plays several structural and metabolic roles that are pivotal for the human body. Various experimental evidence highlights that the homeostatic maintenance of cholesterol metabolism is essential in the brain, especially for differentiation, synaptogenesis and neurotransmission.

The most part of cholesterol in the central nervous system (CNS), occurs through *de novo* synthesis, which specifically occurs in brain cells. Differently from embryogenesis, when neurons autonomously provide for their cholesterol needs, during adulthood neurons abandon the biosynthesis and the requirement of this sterol is supplied by ApoE-rich lipoproteins derived from astrocytes, which remains metabolically active.

Several data evidenced that epigenetic factors can play a key role in the control of metabolic processes, including cholesterol metabolism. In this regard, the involvement of Bromodomain and Extra-Terminal domain (BET) proteins is still unclear.

Thus, the initial objective of this PhD project was to investigate the role of BET proteins in the modulation of lipid homeostasis. Since cholesterol homeostasis is particularly active in hepatic cells, we employed human liver-derived HepG2 cells, a widely accepted experimental model to study lipid metabolism. The role of BET proteins was unraveled by using a loss-of-function approach, by treating HepG2 cells with JQ1, a powerful and selective BET inhibitor. The main results show that BET blockade determined a significant modulation of the proteins involved in lipid biosynthesis, uptake and intracellular trafficking.

Once ascertained that BET blockade by JQ1 modulates cholesterol metabolism, the effects of BET inhibition were evaluated on neuronal differentiation, an important physiological process that leads to the formation of new neurons from neural stem cells (NSC). Interestingly, this process is strongly based on cholesterol metabolism. Therefore, we assessed whether BET inhibition could stimulate neuritogenesis through the modulation of cholesterol homeostasis. To reach this aim, different neuronal-derived cell lines were used as experimental model. Data collected displayed that JQ1-mediated BET inhibition induces neuronal differentiation through a remarkable modulation of cholesterol metabolism. In addition, we found that BET blockade-mediated effects are accompanied by the activation of specific signaling pathways and by autophagy induction.

To strengthen the validity of these results, the pro-differentiating effects of JQ1 were confirmed in isolated NSC and on *in vivo* adult neurogenesis in mice.

The attention was then focused on the study of signaling factors putatively involved in the control of cholesterol homeostasis in brain cells. In this context, neurotrophins constitute a class of growth factors capable of influencing metabolism of different cell types, although they were initially identified as signal molecules closely involved in neuronal survival and differentiation.

Specifically, we examined the impact of nerve growth factor (NGF) in cholesterol homeostasis. Since this metabolic process is highly regulated in astrocytes, we investigated whether NGF could influence cholesterol metabolism in glial cells. To reach this objective, an astrocyte-derived cell line, U373, was employed. Our results show that proteins implicated in cholesterol metabolism network increase following NGF administration in U373 cells. Furthermore, NGF considerably enhances ApoE secretion and extracellular cholesterol content in the culture medium. Since ApoE has been reported to increase the resilience of neurons to oxidative insult, we evaluated the impact of NGF on survival under conditions of oxidative stress. Co-cultures and U373-conditioned medium experiments revealed that NGF treatment efficiently contrasts redox dysbalance in N1E-115 neurons. On the contrary, neuroprotective effects mediated by NGF are abolished when N1E-115 are co-cultured with ApoE-silenced U373 cells. Thereby, these data suggest that NGF modulates cholesterol metabolism in astrocytic cells and exhibits neuroprotection against oxidative stress by promoting glial ApoE secretion.

Collectively, the data collected in this PhD thesis provide new hints about the molecular mechanisms linking the regulation of cholesterol metabolism in brain cells. Clarifying the molecular mechanisms that induce, regulate and maintain brain processes is not only fundamental to better understand the CNS physiology, but also to develop new therapeutic strategies useful for counteracting most neurodevelopmental and neurodegenerative diseases associated to defects in cholesterol metabolism.

INTRODUCTION

1. CHOLESTEROL

1.1 Structure and functions

Cholesterol (Figure 1) is a ubiquitous lipid molecule from the animal Kingdom and its homeostasis is essential for cell metabolism and development. From a biochemical point of view, cholesterol is a 27-carbon sterol containing a four-ring steroid nucleus fused into a rigid, planar conformation. Its structure is characterized by the presence of an unsaturated double bond (C5-C6), a hydroxyl group (C3) and a short aliphatic tail (*Bloch, 1991*). The central tetracyclic nucleus and the chemically apolar side hydrocarbon chain and the polar 3-hydroxyl group make this compound amphipathic. Therefore, in aqueous solution cholesterol forms micellar aggregates, in which only the hydrophilic region is in contact with the solvent (*Schade et al., 2020*).

In biological systems cholesterol is found exclusively in membranes and in complex with other lipids and/or proteins, such as lipoproteins and lipid droplets (*Cortes et al., 2014*).

Given its chemical and structural properties, cholesterol represents the key constituent of numerous physiological processes in animal organisms (*Zhang et al., 2019*).

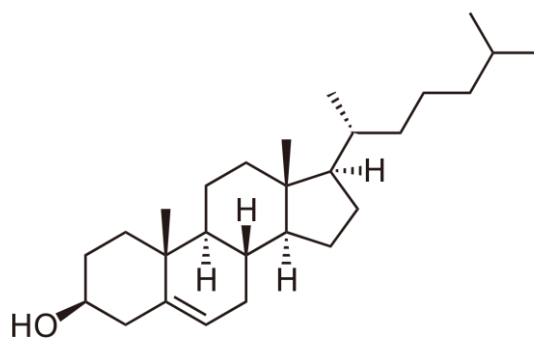


Figure 1. Structure of cholesterol. Cholesterol is a sterol with a unique structure, formed by a central steroid nucleus consisting of four fused rings, an aliphatic tail and a hydroxyl group. Chemically, cholesterol is an amphipathic molecule, capable of forming micellar aggregates in aqueous solution (Modified from Wikipedia).

Cholesterol is the most abundant sterol in animal tissues. Because of its hydrophobic nature, it is an essential component of cell membranes. In the plasma membrane, cholesterol constitutes 20-25% of the total lipids of the bilayer, while the remainder is formed by various phospholipids,

sphingomyelin and glycolipids. Depending on its structure, cholesterol is positioned in proximity of the saturated aliphatic chains of nearby lipids, preventing their crystallization and influencing the biophysical properties of the membrane. The reduced flexibility of these hydrocarbon chains, due to the presence of this sterol, results in a greater packing of lipids in the bilayer with a consequent reduction in membrane fluidity and permeability (*Ikonen, 2008*).

Furthermore, cholesterol guarantees the formation and the functionality of lipid rafts, membrane microdomains that play a primary role in signal transduction and in the maintenance of cell surface polarity (*Simons & Ikonen, 2000*). Numerous experimental evidence suggests that cholesterol is involved in the functioning of many transmembrane proteins. For instance, the direct interaction of this lipid with various types of receptors can lead to increase or decrease in their activity, as well as the facilitation of their structural stability (*Burger et al., 2000; Gimpl et al., 1997*).

In addition to its functional and structural role at membrane level, cholesterol is the precursor of oxysterols, bile acids and all steroid hormones (*Luo et al., 2020*). A cholesterol precursor, 7-dehydrocholesterol, is converted to pre-vitamin D₃ by UV radiation in the skin, and is then further metabolized into calcitriol, the active form of vitamin D (*Warren et al., 2021*).

1.2 Cholesterol metabolism

Animal organisms have developed complex mechanisms to regulate the abundance and distribution of sterols within their cells. In fact, cholesterol homeostasis is one of the most intensely regulated processes in biology and its alterations are cause of numerous pathologies during development and adulthood (*Goedeke & Fernández-Hernando, 2012; Cortes et al., 2014*). High blood cholesterol levels are correlated to the onset of cardiovascular diseases, such as atherosclerosis, coronary heart diseases and hypertension. Moreover, recent studies indicate a potential involvement of cholesterol deregulation in the development of some types of cancer (*Kuzu et al., 2016*).

Defects in cholesterol biosynthesis and metabolism are responsible for different types of disorders such as Smith-Lemli-Opitz syndrome (*DeBarber et al., 2011*), Wolman disease (*Aguisanda et al., 2017*) and Niemann-Pick disease type C (*Pallottini & Pfrieger, 2020*). Alterations in the metabolism of this lipid in the brain are also associated with neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and other old age atypical cognitive deficits (*Dai et al., 2021*).

Considering its pivotal role in a plethora of biological processes, it is not surprising that intracellular cholesterol levels are finely regulated through intricate mechanisms controlling biosynthesis, absorption, efflux and esterification (*Luo et al., 2020*).

1.2.1 Cholesterol biosynthesis

Cholesterol present in human body derives from two sources: it can be absorbed from the diet (300-500 mg/day) or synthesized within various organs (700-900 mg/day). Indeed, almost every cell type is equipped with the enzymatic machinery required to synthesize cholesterol. However, the contribution of different organs in the production of this lipid varies a lot. As an example, in mammals, the liver accounts for the most important cholesterol production, as hepatocytes produce about 50% of total cholesterol found in the whole body. Additionally, other tissues characterized by active cholesterol biosynthesis are the gastrointestinal tract, the skin, the skeletal muscle and the bone marrow (*Repa & Mangelsdorf, 2000; Dietschy, 1984*). Notably, these extra-hepatic tissues can produce considerable quantities of cholesterol, comparable to those synthesized by the liver itself (*Dietschy et al., 1993*).

Cholesterol is biosynthesized from acetyl coenzyme A (CoA) units through about thirty enzymatic reactions (*Xu et al., 2020*).

The initial step involves the formation of acetoacetyl-CoA from two molecules of acetyl-CoA. The condensation of a third acetyl-CoA unit leads to the synthesis of 3 β -hydroxy-3 β -methylglutaryl-CoA (HMG-CoA). 3 β -hydroxy-3 β -methylglutaryl-CoA is converted into mevalonate (MVA) by HMG-CoA reductase (HMGCR), a key enzyme of the biosynthetic pathway. Subsequently, isopentenyl pyrophosphate (IPP) is formed from the MVA through a series of phosphorylation and decarboxylation reactions, constituting the starting structure for the subsequent polymerization steps. The final product of these reactions is the squalene, a 30-carbon compound and the biochemical precursor of sterols.

Finally, squalene undergoes a series of cyclization reactions giving origin to lanosterol, which is subsequently converted in cholesterol (*Russell, 1992*).

As above mentioned, the formation of MVA from HMG-CoA represents the rate-limiting step of the biosynthetic pathway and is catalyzed by HMGCR. This enzyme, as well as others involved in the last stages of cholesterol synthesis, is an integral membrane protein of the endoplasmic reticulum (ER) (*Chang et al., 2006*).

From a structural point of view, HMGCR is a 97 kDa glycoprotein consisting of a cytosolic C-terminal domain containing the catalytic site and a transmembrane N-terminal domain (*Liscum et al., 1985*). The transmembrane domain (sterol-sensing domain, SSD) acts as a sensor for sterol levels in the ER membrane. Being a key enzyme, the activity of HMGCR is finely modulated at transcriptional, translational and post-translational levels through a complex regulatory system mediated by MVA pathway end-products (*Goldstein & Brown, 1990*).

High levels of oxidized and methylated sterols, such as 25-hydroxycholesterol and lanosterol, decrease the expression of HMGCR and increase in its degradation, reducing the enzyme activity up to 90% (*Russell, 1992*).

In particular, the degradation process requires the interaction of HMGCR with one of the two Insulin-induced gene (Insig) proteins, Insig1 and Insig2. When oxysterols and lanosterol accumulate in the cell, Insigs bind to the transmembrane domain of HMGCR, inducing its ubiquitination and subsequent degradation through the proteasome complex (*DeBose-Boyd, 2008*). HMGCR phosphorylation constitutes a further modality of regulation of the enzyme. In the liver, the AMPK enzyme is the main responsible for the inhibitory phosphorylation, thus blocking the biosynthesis of cholesterol in the presence of low intracellular levels of ATP (*Luo et al., 2020; Sharpe & Brown, 2013*). HMGCR dephosphorylation by the protein phosphatase 2A (PP2A) completely restores the activity of the enzyme (*Burg & Espenshade, 2011*).

Long-term regulation of cholesterol metabolism includes the intervention of transcription factors of the SREBP family (sterol regulatory element-binding protein) (Figure 2). In particular, the SREBP-1 protein regulates the expression of genes involved in lipogenesis, while SREBP-2 promotes the transcription of genes responsible for the synthesis of cholesterol (*Goldstein, DeBose-Boyd & Brown, 2006*). SREBP transcription factors are synthesized as inactive precursors bound to the ER membrane through the C-terminal domain, and their activation requires proteolytic cleavage followed by release of the N-terminal portion. In the presence of elevated cholesterol levels, SREBP cleavage-activating protein (SCAP), acts as a sterol sensor, undergoes a conformational change and binds to SREBP-2 and Insigs protein. Consequently, the SCAP/SREBP-2 complex is retained in the ER membrane, preventing the proteolytic activation of the transcription factor. When cholesterol content in the ER membrane is low, Insig proteins dissociate from SCAP, allowing the release of the SCAP/SREBP-2 complex which migrates through vesicle transport to the Golgi apparatus. In the Golgi, SREBP-2 is subjected to proteolytic cleavage by S1P and S2P proteases, leading to the release of the transcriptionally active fragment. SREBP-2 active fragment (nuclear SREBP-2, nSREBP-2) translocates into the nucleus and binds

to the sterol regulatory elements (SRE), promoting the transcription of genes required for cholesterol synthesis and uptake (Corton, 2019; Espenshade & Hughes, 2007).

nSREBP-2 can be phosphorylated by ERK and AMPK, leading to an increase and decrease of its transcriptional activity, respectively (Luo et al., 2020).

Furthermore, the expression of the *srebf-2* gene, encoding the SREBP-2 protein, is positively regulated by nSREBP-2 through a positive feedback mechanism, due to the presence of a SRE region upstream of promoter (Tao et al., 2013).

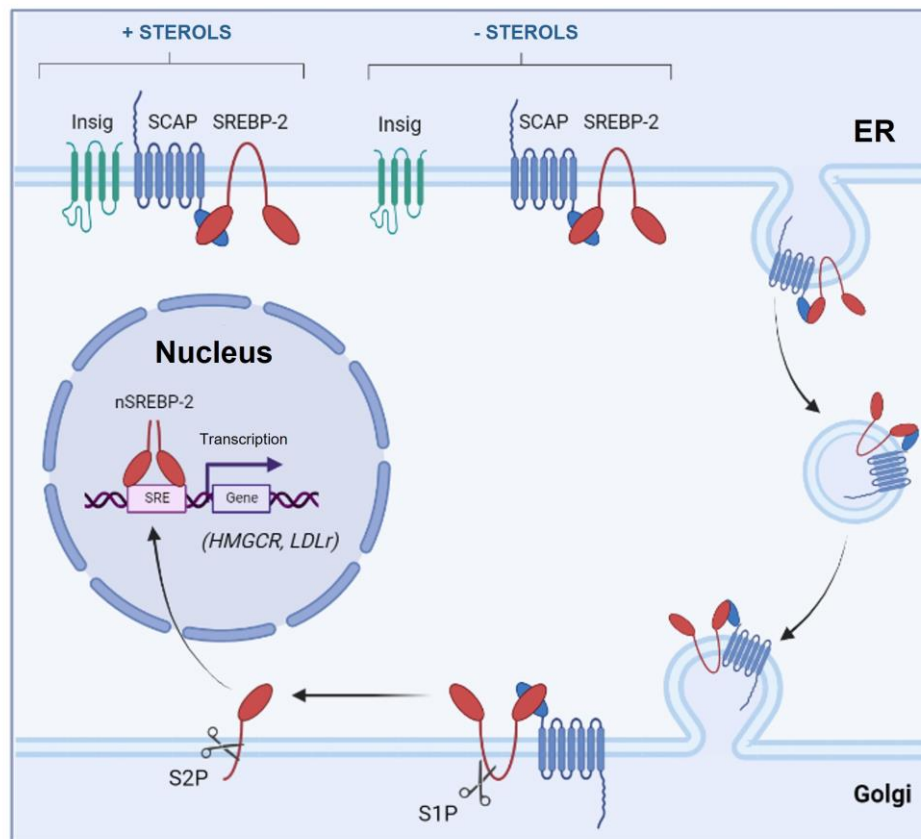


Figure 2. SREBP2-mediated regulation. In presence of cholesterol or oxysterols, SREBP-2/SCAP complex is retained in the ER by Insig. In the absence of sterols, Insig dissociates from SREBP-2/SCAP. Thus, the complex migrates through intracellular vesicles to the Golgi apparatus. Here, the transcriptionally active fragment of SREBP-2 (nSREBP-2) is released from the membrane by means of two proteolytic cleavages mediated by S1P and S2P proteases. In the nucleus, nSREBP-2 binds the SRE sequences present in the promoters of the target genes, inducing their transcription. The figure was created with BioRender.

1.2.2 Cholesterol efflux

Cholesterol efflux is a fundamental process for cellular homeostasis maintenance, as its function is to prevent the excessive accumulation of this lipid within the cell. With a few exceptions represented by hepatocytes and chromaffin cells of the adrenal medulla, most cells are unable to

catabolize cholesterol. Therefore, cholesterol excess must be removed from peripheral cells and transported to the liver through a process known as reverse cholesterol transport (RCT) (Goedeke & Fernández-Hernando, 2012). Once in the liver, cholesterol can be excreted directly in the bile or converted into bile acids and, subsequently, eliminated (Mahley *et al.*, 2006). Free cholesterol efflux from cells represents the sum of different mechanisms: some of them are passive, others dependent on proteins with different characteristics and kinetics (Fielding & Fielding, 2001). High-density lipoproteins (HDL) in plasma and lymph are the main acceptors of cholesterol released by extra-hepatic cells (Chang *et al.*, 2006). Thereby, free cholesterol efflux from the cell membrane to the HDL constitutes the first step of the RCT and, in the case of macrophages, involves four different pathways. Two of them are based on passive and facilitated diffusion; while the remaining ones require the involvement of ABC family (ATP-binding cassettes) transport proteins, in particular ABCA1 and ABCG1 (Phillips, 2014). Cholesterol is sufficiently soluble in water to be exchanged between cell membranes and an acceptor by means of so-called aqueous diffusion. This process includes the desorption of sterol molecules from a water-lipid interface, their diffusion into the aqueous phase of the extracellular medium and, ultimately, adsorption into the HDL particles. The aqueous diffusion mechanism is driven by the cholesterol concentration gradient and is influenced by different physico-chemical parameters (Yancey *et al.*, 2003).

However, the most significant contribution to cholesterol efflux derives from ABC transporters (Figure 3), which represent regulators of this process not only in macrophages but also in other cell types. Particularly, ABCA1 transporter mediates the rate-limiting step in the formation of HDL as well as the maintenance of plasma levels of this lipoprotein (Ikonen, 2008). Apoprotein A-1 (apoA-1) is the primary acceptor of cholesterol transported by ABCA1. This gives rise to HDL particles which, under the action of the lecithin-cholesterol acyltransferase (LCAT) enzyme, become mature and competent to acquire additional cholesterol (Luo *et al.*, 2020). ABCA1 cellular expression is regulated at transcriptional level by liver X receptors (LXRs) and by retinoid X receptors (RXRs). LXRs bind oxysterols, cholesterol oxidized derivatives and form heterodimers with RXRs (Repa & Mangelsdorf, 2000).

The active heterodimeric complex interacts with specific response elements at the DNA level, driving the expression of the *abca1* gene and other genes engaged in the efflux of sterols (Chang *et al.*, 2006).

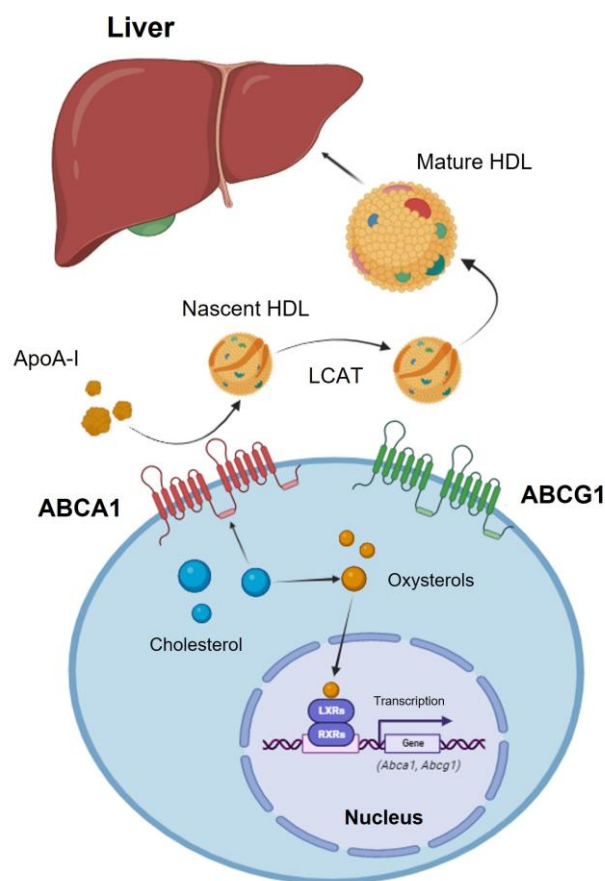


Figure 3. Reverse cholesterol transport. Free cholesterol efflux is mediated by ABC transporters, including ABCA1 and ABCG1. Thanks to the activity of these transmembrane proteins, free cholesterol is transferred to the apoprotein apoA-1, forming nascent HDL. The LCAT enzyme promotes nascent HDL maturation and further cholesterol uptake. Through the bloodstream, mature HDL reaches the liver. The cellular expression of ABCA1 and ABCG1 transporters is transcriptionally regulated by the LXRs and RXRs receptors. LXRs bind oxysterols and form heterodimers with RXRs. The active heterodimeric complex interacts with specific response elements at the DNA level, promoting the expression of the *abca1* and *abcg1* genes. The figure was created with BioRender.

1.2.3 Cholesterol uptake and esterification

Besides *de novo* biosynthesis in different tissues of the body, cholesterol absorption from bile and food sources contributes to the homeostatic maintenance of this lipid (Wang *et al.*, 2017). The process takes place in the small intestine and can be divided into two functionally distinct phases: the uptake, which refers to the entry of cholesterol into the cells of the intestinal epithelium, and the assimilation, which indicates the transfer of sterol into the lymphatic system (Nakano *et al.*, 2019). Therefore, the intestinal absorption of cholesterol is configured as a multiphase event, the efficiency of which depends on the net contribution between the efflux and uptake of lipid molecules through the enterocyte membrane (Lammert & Wang, 2005). Although the small intestine can absorb cholesterol from the lumen, the process is mostly concentrated at the level of

the duodenum and proximal jejunum. Cholesterol absorption begins in the stomach, where the food bolus is mixed with gastric and salivary enzymes and then transported to the duodenum, where bile and pancreatic juices are excreted (Wang, 2003). Some products deriving from lipolytic activity, including free cholesterol, are relatively insoluble in the aqueous phase of the intestinal lumen. Thus, the solution of bile salts, with the addition of phospholipids and monoacylglycerol, significantly increases their solubility, leading to the formation of micellar aggregates (Martini & Pallottini, 2007). In fact, the micelles represent a means of reserve and transport of cholesterol to intestinal epithelial cells, in which the uptake of this lipid in monomeric form occurs (Wang, 2007). NPC1L1 protein (Niemann-Pick type C1-Like 1) is a key mediator in cholesterol uptake via clathrin-mediated endocytosis. NPC1L1 is a glycosylated integral protein expressed on the apical surface of enterocytes and on the hepatocyte membrane (Luo *et al.*, 2019). Moreover, it contains a sensor domain for sterols (SSD) which is also present in other crucial regulators of cholesterol homeostasis, including HMGCR and SCAP (Altmann *et al.*, 2004). NPC1L1 primarily resides in the endocytic recycling compartment (ERC) and, in the presence of reduced cholesterol levels, rapidly translocates towards the plasma membrane to promote its uptake (Figure 4) (Yu *et al.*, 2006).

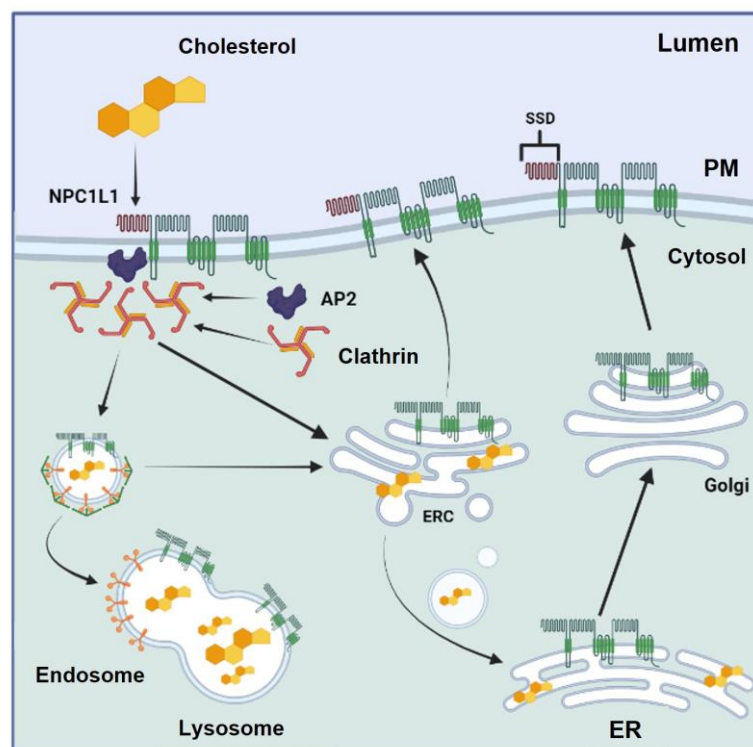


Figure 4. NPC1L1-mediated cholesterol uptake. The transmembrane protein NPC1L1 is synthesized in the ER and, subsequently, is directed to the plasma membrane via the Golgi apparatus. When cholesterol levels are low, NPC1L1 is localized to the apical membrane. Free cholesterol directly binds to the NPC1L1 N-terminal domain, which hosts a sterol sensor domain (SSD). The binding results in a conformational change of the protein and in the recruitment of the clathrin/AP2 complex. The receptor is internalized via

coated vesicles and moves through the endo-lysosomal compartment to be degraded. However, the majority of NPC1L1 is directed to the ERC and, subsequently, to the plasma membrane for a new round of endocytosis. Free cholesterol can dissociate from the receptor in late endosomes or in the ERC and then be transported to the ER for esterification. The figure was created with BioRender.

Inside the enterocytes, the absorbed cholesterol is transported to the endoplasmic reticulum, where it undergoes an esterification process. Cholesteryl esters are then incorporated into the nascent chylomicron particles (Lammert & Wang, 2005; Martini & Pallottini, 2007). Chylomicrons are secreted into the lymphatic system and then released into the bloodstream. In the liver, cholesterol in chylomicrons is incorporated into very low-density lipoprotein (VLDL) for distribution to peripheral tissues. VLDLs are converted into LDL following a partial deprivation of the triglyceride content, thanks to the action of the enzyme endothelial lipoprotein lipase (LPL) (Zannis *et al.*, 2004). The low-density lipoprotein receptor (LDLr) present on the cell surface removes LDL in the plasma by a process of receptor-mediated endocytosis (Figure 5) (Hobbs *et al.*, 1992).

LDLr activity is particularly important as the accumulation of LDL in the blood is the main risk factor for the development of cardiovascular diseases (Borén *et al.*, 2020). Furthermore, mutations in the gene encoding LDLr cause familial hypercholesterolemia, an autosomal dominant disease characterized by high blood cholesterol levels (Goldstein & Brown, 2009). At transcriptional level, the expression of the *ldlr* gene is regulated by the transcription factor SREBP-2 (Goldstein *et al.*, 2006). The mature LDLr is a transmembrane glycoprotein formed by five structurally and functionally distinct modules: an N-terminal binding domain, a large region homologous to the epidermal growth factor precursor (EGFP), an O-glycosylated enriched domain, a single transmembrane portion and a short C-terminal tail that hosts the NPxY internalization motif (Jeon & Blacklow, 2005).

After the binding to circulating lipoproteins, LDL-LDLr complex is initially internalized in clathrin-coated vesicles and, subsequently, reaches the endosomal compartment (Alfonso *et al.*, 2018). In early endosomes, the acid pH ($\text{pH} < 6$) determines a conformational change of LDLr causing its dissociation from LDL (Rudenko *et al.*, 2002).

Then, LDLr can be recycled to the plasma membrane via vesicular transport mechanisms mediated by small GTPases of the Rab family, such as Rab8 and Rab11 (Goedeke & Fernández-Hernando, 2012). Alternatively, LDLr can be directed to lysosomal degradation when it is not dissociated from LDL or upon interaction with PCSK9 (proprotein convertase subtilisin/kexin type 9), a serine protease mainly expressed in the liver (Islam *et al.*, 2022).

The esterified cholesterol in LDL passes through the endo-lysosomal system to be hydrolyzed by lysosomal acid lipase, thus releasing free cholesterol (*Brown et al., 1976*).

Finally, free cholesterol is exported from the lysosomal lumen to other sub-cellular compartments through to the concerted action of Niemann-Pick proteins of type C1 (NPC1), type C2 (NPC2) and lysosome-associated membrane protein 2 (LAMP2) (*Li & Pfeffer, 2016*). The importance of NPC1 and NPC2 in the intracellular cholesterol transport out of the endosomal system is evidenced by the fact that mutations in the *npc1* and *npc2* genes cause excessive accumulation of this lipid in lysosomes: the clinical manifestation is Niemann-Pick type C disease, a lipidosis characterized by hepatomegaly and progressive neurodegeneration (*Scott & Ioannou, 2004*).

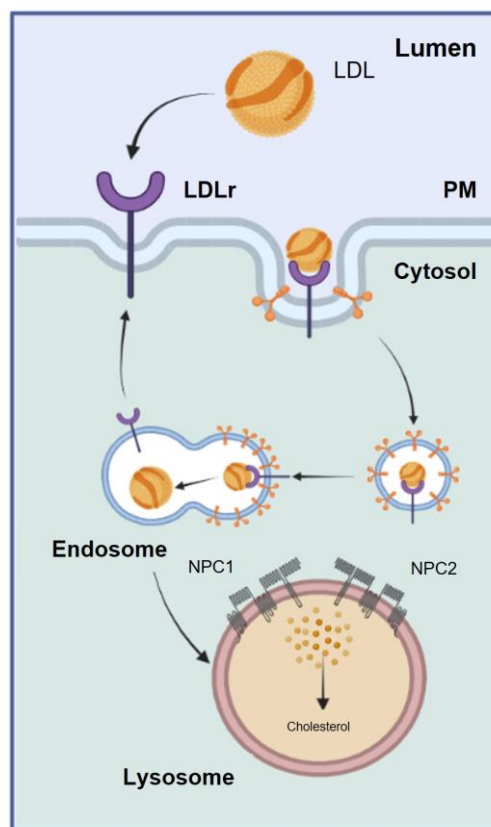


Figure 5. LDLr-mediated cholesterol uptake. LDLr on the cell surface removes LDL in plasma by receptor-mediated endocytosis process. Once circulating lipoproteins bind to LDLr, the LDL-LDLr complex is internalized in clathrin-coated vesicles, which fuses to form endosomes. In early endosomes, acidic pH results in the dissociation of the LDL/LDLr complex. LDLr, at this point, can be recycled towards the plasma membrane. Once free cholesterol is formed, it can be exported to other cellular compartments thanks to the NPC1 and NPC2 proteins. The figure was created with BioRender.

The accumulation of large amounts of cholesterol in cells can be deleterious because it triggers mechanisms of cellular toxicity, including the formation of oxysterols and the activation of pro-apoptotic signaling pathways (*Tabas, 2002*).

In order to prevent this accumulation, about half of the cholesterol that enters the enterocytes is transported to the ER, where it is esterified by the enzyme acyl-CoA:cholesterol acyltransferase

(ACAT) (Wang, 2003). Mammals, including humans, have two isoenzymatic forms of ACAT: ACAT1 and ACAT2 (Rogers *et al.*, 2015).

While ACAT1 is localized in many tissues, the expression of ACAT2 protein is limited to two cell types: enterocytes of the small intestine and hepatocytes (Parini *et al.*, 2009).

Cholesterol, interacting with ACAT1, acts as an activator and determines a conformational change of the protein that facilitates the binding with the substrate and enhances the catalytic activity of the enzyme (Liu *et al.*, 2005). When compared to ACAT1, experimental data suggest that ACAT2 is more efficient in esterifying 25-hydroxycholesterol and bile acid derivatives than cholesterol itself (Cho *et al.*, 2003). Finally, both enzymes are expressed in macrophages of atheromatous lesions, suggesting their involvement in the pathogenesis of atherosclerosis and their use as potential therapeutic targets for the treatment of this disease (Meiner *et al.*, 1996; Kusunoki *et al.*, 2001).

1.3 Epigenetic regulation of cholesterol metabolism

In the last few years, scientific evidence suggests that epigenetic mechanisms may exert pivotal roles in regulating cholesterol homeostasis (Meaney, 2014).

Several *in vitro* and *in vivo* studies have shown that the genes involved in cholesterol biosynthesis are under epigenetic control. By an *in vitro* approach, it has been observed that overexpression of histone deacetylase 3 (HDAC3) in CHO cells leads to a 10-fold reduction in the rate of cholesterol synthesis (Villagra *et al.*, 2007). To preclude a *srebf-2*-mediated effect, the SRD-13A cell line, which cannot cleave SREBP-2 and therefore is unable to produce cholesterol, was used (Rawson *et al.*, 1999). In this case, HDAC overexpression only produced a slight difference if compared to a control cell line, indicating that the effect was mainly mediated by epigenetic mechanisms. Furthermore, Villagra and colleagues demonstrated that gene expression of lanosterol synthetase, the enzyme that catalyzes the cyclization of isoprenoid 2,3-oxidosqualene to lanosterol, was strongly repressed by HDAC3 expression and activated by trichostatin A (TSA) treatment, a class I and II HDAC inhibitor (Villagra *et al.*, 2007). Coherently, using an *hdac3* knockout mouse, it was demonstrated that the loss of HDAC3 caused a de-repression of cholesterol biosynthesis and secretion by the liver, resulting in a marked increase in various genes involved in the synthesis of this sterol (Knutson *et al.*, 2008).

Additionally, several genes of cholesterol synthesis resulted to be downregulated following TSA treatment in HepG2 and F9 cell lines. Since *srebf-2* was also decreased, this may explain the

observed changes in cholesterol synthesis in the experimental models studied (*Chittur et al., 2008*). Drzewinska et al. reported that 24-dehydrocholesterol reductase (dhcr24) was induced following both TSA and 5-azacytidine (5AZA, a substance with high DNA demethylating activity) treatment in almost all cell lines used. All these data indicate that epigenetic mechanisms are fundamental for the regulation of cholesterol metabolism and that the sensitivity to epigenetic regulation varies according to the cell type (*Drzewinska et al., 2011*).

Protein deacetylases belonging to the SIRT family also affect cholesterol homeostasis. Indeed, a primary rat neuronal culture, following treatment with SIRT2 inhibitors, showed a reduction in the expression of several genes of cholesterol synthesis and in the amount of cholesterol compared to control cells (*Luthi-Carter et al., 2010*). Conversely, in *sirt2* knockout mice, Bobrowska et al. did not confirm these data since there were no changes in the transcriptional levels of the cholesterologenic genes (*Bobrowska et al., 2012*). However, these researchers had analyzed brain regions with different cell types and any differences could be muffled by a greater presence of cell types in which no changes occurred. This could be explained by the fact that the expression of genes involved in sterol homeostasis in neurons changes according to the age and, in particular, in the postnatal period (*Ohyama et al., 2006*).

Sirt6 also appears to be involved in cholesterol homeostasis. The amount of cholesterol was shown to decrease following ectopic overexpression of *sirt6* in HepG2 cells. This regulation was caused by the effects of *sirt6* on *srebf-1* and *srebf-2* through a combination of direct transcriptional regulation, inhibition of proteolytic release of the SREBP nuclear form, and promotion of SREBP phosphorylation by AMPK (*Elhanati et al., 2013*). Subsequently, Tao and colleagues highlighted the key role of forkhead box O3 (FOXO3) in the recruitment of *sirt6* to the *srebf-2* promoter, resulting in histone deacetylation and reduced expression of cholesterol biosynthesis genes. Furthermore, the effects of *sirt6* overexpression on cholesterol content were also observed *in vivo* (*Tao et al., 2013*).

There are few data about the epigenetic mechanisms that regulate genes implicated in cholesterol uptake. Malhotra and colleagues described a key role of *npc111* promoter methylation in the mouse gastrointestinal tract. NPC1L1 expression is known to be restricted to the gastrointestinal tract, particularly the small intestine. The low expression in the colon appears to be due to extensive promoter methylation and treatment with 5AZA resulted in a significant dose-dependent increase in *npc111* expression in the colon. Furthermore, methyltransferases 1 and 3B have been shown, through gene knockdown, to be involved in maintaining the hypermethylated state in the colon (*Malhotra et al., 2014*). There are also few data on the epigenetic regulation of ABCG5 and

ABCG8. However, Imai et al. observed that the common promoter is acetylated and unmethylated, at least in the liver (Imai et al., 2009).

Regarding cholesterol storage, the available data on the regulation of ACAT1 and ACAT2 are very limited. It was identified that the *acat2* promoter was methylated to a different extent in lung adenocarcinoma tumor cells (Tessema and Belinsky, 2008). Liang and colleagues demonstrated a modest decrease in *acat1* promoter methylation following homocysteine administration, resulting in increased *acat1* transcriptional levels and cholesteryl esters content (Liang et al., 2013). Finally, Devlin et al. observed a decreased hepatic expression of ACAT2 in mice affected by hyperhomocysteinemia in the context of altered DNA methylation (Devlin et al., 2010). Thus, although still insufficient, the available data suggest that cholesterol esterification is probably subjected to epigenetic regulation.

2. CHOLESTEROL IN THE CENTRAL NERVOUS SYSTEM

2.1 Cholesterol functions in the CNS

In humans, the central nervous system (CNS) contains the highest amount of cholesterol if compared to any other tissue (~23 mg/g). Indeed, although the CNS constitutes only 2% of body weight, it contains about 25% of the total cholesterol present in the whole body (Dietschy & Turley, 2004).

Most of cholesterol (>99,5%) in the nervous system is found in free form in two compartments: the plasma membrane of neurons and astrocytes, and the myelin sheaths (Björkhem & Meaney, 2004). The presence of myelin is essential for neuronal physiology, as the discontinuous isolation of the axon allows the rapid propagation of the action potential (Orth & Bellosta, 2012).

Hence, cholesterol plays a vital role in neuronal physiology, both during development and in adult life (Zhang & Liu, 2015).

Furthermore, cholesterol is indispensable for the formation of dendrites and directly stimulates the differentiation of presynaptic terminals. Although neurons can survive and grow in the absence of cholesterol, specific phases of neuronal differentiation depend on the supply of this lipid (Goritz et al., 2005).

This sterol is also important in synaptogenesis. The membranes of synaptic vesicles contain higher amounts of cholesterol than those of other intracellular organelles. Additionally, the assembly, transport and exocytosis of these vesicles are tightly linked to membrane microdomains rich in cholesterol (*Fester et al., 2009*).

Numerous studies have demonstrated that neurotransmitter receptors and other postsynaptic components are associated with these microdomains, suggesting that adequate cholesterol levels are essential for their structural and functional organization (*Pfriege, 2003*).

Indeed, the deficiency of this sterol in neurons compromises the exocytosis of synaptic vesicles, neuronal activity, and neurotransmission, leading to the degeneration of dendritic spines and synapses (*Linetti et al., 2010; Liu et al., 2010*).

Therefore, alterations in cholesterol metabolism are associated with several pathological conditions of the CNS. These include neurodegenerative and neurodevelopmental diseases, such as Alzheimer's disease, Parkinson's disease, Niemann-Pick disease type C, Huntington's disease, amyotrophic lateral sclerosis (ALS) and Rett syndrome (*Jin et al., 2019; Segatto et al., 2019*).

2.2 Cholesterol metabolism in the CNS

Cholesterol in the CNS is synthesized almost entirely *in situ*, since the blood-brain barrier (BBB) prevents direct exchange of sterols between the blood and the brain (*Dietschy & Turley, 2001*). It has been shown that brain endothelial cells are involved in cholesterol uptake from the bloodstream via a transcytosis mechanism mediated by LDLr receptors. However, this process is of minimal relevance (*Dehouck et al., 1997*).

Conversely, several hydroxylated metabolites derived from cholesterol, including 24-hydroxycholesterol (24-OHC) and 27-hydroxycholesterol (27-OHC), are able to cross the BBB, probably regulating the homeostasis of this lipid (*Saeed et al., 2014*). Most of cholesterol in the CNS is synthesized between the perinatal period and adolescence, when the axons are surrounded by myelin sheaths. In the adult brain, at the end of the myelination process, cholesterol synthesis and its turnover are reduced to minimum levels (*Morell & Jurevics, 1996*).

Consequently, cholesterol in the nervous system of an adult individual has an extremely long half-life (~ 5 years) (*Björkhem et al., 1998*).

From these observations, it is clear that, in the presence of an intact BBB, CNS cholesterol metabolism is separated from the rest of the organism. The *de novo* biosynthesis of this lipid occurs through the MVA pathway, formed starting from acetyl-CoA units, through the activity of

HMGCR. A series of enzymatic reactions then converts MVA into squalene and, finally, into cholesterol (Segatto *et al.*, 2012).

Neurons and astrocytes, starting from squalene, lead to the formation of various types of sterols. In particular, neurons mainly synthesize sterols via the Kandutsch-Russel pathway, including lanosterol and 7-dehydrocholesterol, while astrocytes mainly produce precursors of the Bloch pathway, such as desmosterol (Nieweg *et al.*, 2009).

Generally, nerve cells synthesize less sterols when compared to glial cells (Zhang & Liu, 2015). Additionally, *in vitro* studies have shown that astrocytes produce two to three times more cholesterol compared to neurons or fibroblasts (Saito *et al.*, 1987). According to a widely accepted hypothesis, during embryogenesis, neurons autonomously synthesize cholesterol to ensure their survival. Knockout mice for the squalene synthetase (SQS), the enzyme responsible for the synthesis of squalene, have small brains and are affected by neonatal mortality, as a result of cholesterol depletion (Saito *et al.*, 2009).

Nevertheless, in the postnatal period neurons reduce or abandon cholesterol biosynthesis, which is imported by astrocytes via lipoproteins. One possible explanation for this phenomenon relies in the enormous energy expenditure required for the synthesis of sterols. Therefore, neurons abandon cholesterol synthesis to preserve metabolic energy employed for the generation of action potentials (Pfrieger, 2002).

The dependence of neurons from glia-derived cholesterol is supported by published reports showing that most of the synapses in the CNS is formed only after astrocyte differentiation (Pfrieger & Barres, 1996).

Horizontal transport between the two cell types begins in astrocytes, where newly synthesized cholesterol is translocated to the plasma membrane and incorporated into LDL through the transporters of the ABC family (Oram & Heinecke, 2005). ABCA1 and ABCG1 are highly expressed in the CNS, where they play an essential role in the formation of ApoE-containing lipoproteins (Tachikawa *et al.*, 2005). ABCA1 is expressed at both neurons and glial cells and catalyzes the transfer of cholesterol to lipid-free apolipoproteins, such as ApoE, to form nascent LDL particles. The latter are then completely lipidated in a second step of efflux mediated by ABCG1 (Vaughan & Oram, 2006). According to this notion, cholesterol efflux from astrocytes is reduced in mice lacking ABCA1, leading to an intracellular accumulation of this lipid and decreased lipidation of ApoE (Hartz & Bauer, 2011). The expression of these transporters and ApoE is regulated by the transcription factors LXRs, which are sensitive to increased cytoplasmic levels of oxidized cholesterol metabolites, such as 24-OHC (Koldamova & Lefterov, 2007). ApoE is the major apolipoprotein of the CNS and it is a constituent of the lipoproteins that transport

cholesterol between nerve and astrocyte cells. Under physiological conditions, ApoE is mainly produced from astrocytes and is present in the cerebrospinal fluid (CSF) of humans and rodents (*Fernandez et al., 2019*). The binding of ApoE to lipoprotein receptors is able to modulate the development of neuronal extensions and synaptic plasticity (*Beffert et al., 2003*). Lipoprotein uptake into the CNS is mediated by several members of the LDL receptor family and, among these, LDLr and LRP1 (LDL receptor-related protein 1) are the main proteins capable of binding and internalizing ApoE-rich lipoprotein particles (*Mahley, 2016*). Once endocytosed, lipoproteins are processed through the endolysosomal system, leading to the formation of free cholesterol. The latter reaches the various compartments sub-cellular through the activity of NPC1 and NPC2 proteins (*Pfriegeer & Ungerer, 2011*). This redistribution process is particularly relevant in neurons, as the NPC1 or NPC2 dysfunction is at the root of the typical neurological symptoms of NPC disease (*Karten et al., 2006*).

Considering its crucial involvement neuronal physiology, cholesterol levels in the CNS must be carefully regulated to prevent abnormal accumulation. Excess cholesterol is subject to esterification by the ACAT1 enzyme and is stored within lipid droplets (*Bryleva et al., 2010*). Particularly, ACAT1 appears to be more active in neurons than in glial cells (*Sakashita et al., 2000; Karten et al., 2006*).

Alternatively, excess cholesterol can be secreted from the CNS via two different mechanisms. The first, although not very relevant, is based on the capacity of astrocytes to directly release minimal amounts of ApoE-cholesterol (1-2 mg/day) into the cerebrospinal fluid (*Pitas et al., 1987*). The second mechanism of secretion, quantitatively more important, is the elimination of 24-OHC, a cholesterol metabolite synthesized by the enzyme CYP46A1 (*Gamba et al., 2021*). This enzyme is selectively expressed in neurons and is particularly abundant in some neuronal subtypes, such as the pyramidal cells of the cortex and the Purkinje cells of the cerebellum (*Lund et al., 2003*). Being more hydrophilic than cholesterol, 24-OHC can diffuse through the BBB (6-7 mg/day) into the bloodstream driven by a concentration gradient. Next, 24-OHC is transported to the liver to be further converted into bile acids and eliminated from the body by feces (*Björkhem, 2006*).

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PURPOSE OF THE RESEARCH

Cholesterol plays different structural and metabolic roles that are vital for the human body. Particularly, cholesterol is essential to ensure proper functioning of the CNS, as it plays a crucial role in differentiation, synaptogenesis, and neurotransmission (*Suzuki et al., 2007; Lecis and Segatto, 2014*). Cholesterol metabolism in the brain significantly differs from that of other tissues and relies on *de novo* biosynthesis, because of the BBB that prevents cholesterol uptake from the bloodstream (*Dietschy & Turley, 2001*). This *de novo* biosynthesis is finely regulated in different brain cells according to developmental stage. During embryogenesis, before astrocyte differentiation, neurons autonomously provide for cholesterol synthesis. Conversely, during the postnatal period, neurons suppress their cholesterol synthesis to save energy for the generation of action potentials and satisfy their cholesterol demand through lipoproteins derived from astrocytes, in which this sterol production remains particularly intense (*Pfriege, 2002, Pfriege & Ungerer, 2011*). Therefore, alterations in the homeostatic maintenance of this sterol can contribute to the onset of various neurodevelopmental and neurodegenerative diseases, such as Smith-Lemli-Opitz syndrome (*DeBarber et al., 2011*), Niemann-Pick disease type C (*Pallottini & Pfriege, 2020*), Alzheimer's disease, Parkinson's disease, and Huntington's disease (*Dai et al., 2021*).

A better understanding of the molecular mechanisms underlying the regulation of this important metabolic pathway is undoubtedly useful for identifying new molecular targets that can be employed to design promising pharmacological approaches.

Even though the regulation of cholesterol metabolism is well established, several modulatory mechanisms are poorly characterized, and ongoing research continuously sheds light on novel players of cholesterol homeostasis. In the last few years, different findings highlighted that epigenetic factors may exert pivotal roles in the control of cholesterol homeostasis (*Meaney, 2014*). In this context, the role played by Bromodomain and Extra-Terminal domain (BET) proteins is still elusive (*Tonini et al., 2020*). The BET protein family comprises four members: bromodomain-containing protein 2 (BRD2), bromodomain-containing protein 3 (BRD3), bromodomain-containing protein 4 (BRD4), and bromodomain testis associated protein (BRDT). BRD2, BRD3, and BRD4 are expressed in nearly all cells and tissues, whereas BRDT is exclusively expressed in testis (*Jones et al., 1997; Taniguchi, 2016*).

BET proteins are epigenetic readers recruited to the chromatin which recognize acetylated histones and, in turn, regulate gene expression (*Wang et al., 2015*). Recent evidence revealed that BET proteins may play crucial roles in the homeostasis maintenance of the CNS, by regulating the

expression of multiple genes including neurotransmitter receptors, ion channels, and neurotrophic factors (Korb *et al.*, 2015; Sartor *et al.*, 2015; Sullivan *et al.*, 2015; Magistri *et al.*, 2016; Duan *et al.*, 2020). Furthermore, BET proteins display a high affinity for histone modifications associated with learning and memory processes (Umehara *et al.*, 2010a; Umehara *et al.*, 2010b; LeRoy *et al.*, 2012).

BET proteins are very interesting targets for biomedical research, as their activity can be pharmacologically inhibited by powerful and selective inhibitors (Segatto *et al.*, 2017).

Thus, the initial section of the thesis is addressed to the study of BET proteins in the modulation of cholesterol homeostasis. Since cholesterol metabolism is particularly active in the liver, a proof-of-concept study was firstly carried out in a hepatic cell line. In particular, we employed a loss-of-function approach using JQ1, a potent and specific BET inhibitor.

Subsequently, the effect of BET inhibition was assessed in different experimental models of neuronal differentiation. This important physiological process is involved in the development and plasticity of nervous tissue, and strongly relies on cholesterol homeostasis (Cartocci *et al.*, 2016). Thus, our hypothesis was to evaluate whether BET inhibition could promote neurite outgrowth through the modulation of cholesterol metabolism. N1E-115, a mouse neuroblastoma cell line, was used as model system. N1E-115 are commonly used in the study of neuronal differentiation and function. In fact, under appropriate stimuli, these cells exit the cell cycle and differentiate, assuming a neuronal morphology. The neuronal features assumed by differentiated N1E-115 are not exclusively morphological, but also functional, since these cells can develop mature synaptic terminals, produce neurotransmitters and generate action potentials (Kranenburg *et al.*, 1995). In addition, some of the results obtained have been summarized in the NSC-34 and ND7/23 cells, two hybridoma cell models frequently used to study neuritogenesis. To strengthen the validity of the results obtained in immortalized cell lines, the effects mediated by BET inhibition were also tested on isolated murine neural stem cells (NSC) and in *in vivo* adult neurogenesis in mice.

The second part of this thesis is dedicated at identifying novel endogenous factors involved in the control of cholesterol metabolism in brain cells. As already reported, cholesterol metabolism in the CNS relies on a complex intercellular transport between astrocytes and neurons which guarantees the proper homeostasis maintenance. Although this mechanism is well described, endogenous molecules capable of influencing cholesterol biosynthesis in astrocytes and its deliver to neurons are not known. Neurotrophins are a family of growth factors particularly devoted to neuronal survival and differentiation. In addition, recent evidence reveals that neurotrophins may also affect metabolic processes.

To better understand the role of neurotrophins in the homeostatic maintenance of cholesterol in the CNS, a detailed study of literature data was carried out. This intense study resulted in the publication of a review article focused on this topic (*Colardo et al., 2021*). Literature consultation highlighted that few information is available about the role of nerve growth factor (NGF), the progenitor of neurotrophins, in the regulation of cholesterol homeostasis. Therefore, the role of NGF in the control of cholesterol metabolism in astrocytes was assessed in this PhD thesis. We also evaluated whether NGF, by modulating glial cholesterol, could indirectly influence neuritogenesis and resilience of neuronal cells subjected to oxidative stress (*Colardo et al., 2022*). To achieve these goals, U373, an astrocytoma cell line, was used as experimental model. U373 cell line is widely employed for the study of astrocyte metabolism, being easy to manipulate and presenting phenotypic and functional characteristics typical of primary astrocytes (*Volkhardt et al., 2002*).

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ARTICLES CONCERNING THE PhD PROJECT



Article

Inhibition of Bromodomain and Extraterminal Domain (BET) Proteins by JQ1 Unravels a Novel Epigenetic Modulation to Control Lipid Homeostasis

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Abstract: The homeostatic control of lipid metabolism is essential for many fundamental physiological processes. A deep understanding of its regulatory mechanisms is pivotal to unravel prospective physiopathological factors and to identify novel molecular targets that could be employed to design promising therapies in the management of lipid disorders. Here, we investigated the role of bromodomain and extraterminal domain (BET) proteins in the regulation of lipid metabolism. To reach this aim, we used a loss-of-function approach by treating HepG2 cells with JQ1, a powerful and selective BET inhibitor. The main results demonstrated that BET inhibition by JQ1 efficiently decreases intracellular lipid content, determining a significant modulation of proteins involved in lipid biosynthesis, uptake and intracellular trafficking. Importantly, the capability of BET inhibition to slow down cell proliferation is dependent on the modulation of cholesterol metabolism. Taken together, these data highlight a novel epigenetic mechanism involved in the regulation of lipid homeostasis.

Keywords: BET proteins; cell proliferation; cholesterol; epigenetics; HMGCR; JQ1; LDLr; lipid metabolism; SREBP; TMEM97

1. Introduction

The homeostatic regulation of lipid metabolism is essential for the maintenance of key cellular processes involved in a plethora of biological functions. Fatty acids constitute the major energy source and are fundamental constituents of cell membranes [1]. In addition, they serve as substrates for cellular phospholipases, which convert these compounds into pivotal signalling factors implicated in anti- and pro-inflammatory actions [2]. Similarly, cholesterol exerts both structural and functional roles, being the precursor of steroid hormones, vitamin D and bile acids, and regulating the assembly of specialized membrane microdomains called lipid rafts and caveolae [3].

During last decades, it has become increasingly clear that lipid homeostasis is crucial for cell growth and proliferation [4,5], as membrane biosynthesis requires the coordinated assembly of different lipid species during cell division, involving the concerted regulation of lipid biosynthesis, uptake, subcellular localization and turnover [6]. For these reasons, the body employs an intricate homeostatic network to regulate the availability of lipids for cells and tissues. This network mainly

operates in the liver, where the major part of lipid metabolism takes place [3]. Hepatic cells are the principal site for lipid biosynthesis, determined by the establishment of a complex series of enzymatic reactions; in particular, 3-hydroxy-3-methylglutaryl Coenzyme A reductase (HMGCR) and Acyl Coenzyme A carboxylase (ACC) represent the key and rate-limiting enzymes for cholesterol and fatty acid synthesis, respectively [7,8]. Considering their central role in lipid biosynthesis, both HMGCR and ACC are tightly regulated at both short- and long-term levels. Short-term regulation is controlled by phosphorylative events, which negatively affect the activation state of the enzymes [9]. Conversely, the long-term transcriptional regulation of the protein machinery involved in lipid metabolism is mediated by sterol regulatory element binding proteins (SREBPs). When intracellular sterol content is reduced, SREBPs precursors are proteolytically processed to form the NH(2)-terminal fragment that enters the nucleus (nuclear, nSREBP) and induces the transcription of genes coding for lipogenic enzymes, such as HMGCR and ACC [9,10]. Notably, SREBPs also promote the extracellular uptake of lipoprotein-derived lipids by eliciting the transcription of lipoprotein receptors, such as low density lipoprotein receptor (LDLR) and scavenger receptor class B type 1 (SR-B1) [11,12].

Lipid abnormalities play a crucial role in a plethora of pathological conditions, such as cardiovascular diseases, neurodevelopmental alterations, neurodegenerative and lipid storage disorders [13–15]. Despite the widespread use of lipid-lowering medications, effective pharmacological approaches are still lacking for several lipid metabolism-related disorders. Thus, there is still a need to better dissect the regulatory mechanisms of lipid homeostasis in order to identify novel molecular targets that could be useful for designing promising therapeutic treatments.

Bromodomain and extra-terminal domain (BET) proteins, comprising BRD2, BRD3 and BRD4, are epigenetic readers recruited to the chromatin by the presence of acetylated histones, thereby regulating gene expression [16]. The involvement of these epigenetic sensors has been extensively characterized in cancer and inflammation, because of their incontrovertible role in the transcriptional modulation of oncogenes and mediators of the immune response [17,18]. BET proteins attracted considerable interest in biomedical research, as they are extremely druggable by potent and specific inhibitors. In this context, BET inhibition exerts anti-proliferative activity in different cancer cells and shows outstanding anti-inflammatory properties in a number of physiopathological conditions [17,19,20]. Recently, experimental evidence also demonstrated that the activity of BET proteins may be extended to the regulation of metabolic processes. For instance, BET inhibition strongly affects protein homeostasis, autophagy induction, reactive oxygen species (ROS) and glucose metabolism [17,21–24]. Conversely, the prospective role exerted by BET proteins on lipid metabolism is still poorly characterized. Microarray analysis suggested that the BET inhibitor RVX-208 induces changes in ApoA1 and high density lipoprotein (HDL) levels [25]. Coherently, overexpression of BET proteins increased cholesterol biosynthesis [26]. Furthermore, it has been observed that BET inhibition efficiently counteracted plasma low density lipoprotein (LDL) alterations in a mouse model of cancer cachexia [17]. Despite this evidence, no studies systematically addressed the involvement of BET proteins in the modulation of the main proteins and enzymes controlling the regulatory machinery of lipid metabolism. A better comprehension of these mechanisms is essential to identify novel physiological regulatory pathways and to select innovative therapeutic targets. Here, we provide a proof of concept study, aimed at evaluating the putative role of BET modulation on lipid homeostasis. To reach this objective, we took advantage of a loss-of-function approach by using JQ1 as a potent and specific inhibitor of BET protein activity [27]. The effects induced by BET inhibition were mainly evaluated in HepG2 cell line, a human liver-derived cell culture model widely used to assess lipid homeostasis [28–30].

2. Results

2.1. BET Inhibition Decreases Lipid Content in HepG2 Cells

In order to evaluate the putative involvement of BET inhibition on lipid homeostasis, the effect of JQ1 on cellular lipid content was firstly assessed. HepG2 cells were treated with JQ1 for 48 hours,

and Oil Red O staining was employed as a widely used and accurate method to measure neutral lipids [31]. Descriptive evaluation highlighted that the number of lipid droplets, as well as their size, appeared reduced in JQ1-treated cells (Figure 1A). This result corroborated the quantitative assessment of lipid content estimated by Oil Red O absorbance, which showed a significant five-fold decrease upon BET inhibition (Figure 1B). Coherently, immunofluorescence intensity of the lipid droplet marker perilipin-2 (Plin2) was found to be lower 48 hours after pharmacological BET blockade (Figure 1C). Notably, JQ1 was also effective in reducing the amount of intracellular cholesterol, as observable by filipin staining and quantification (Figure 1D). Thus, BET inhibition significantly lowers lipid content in HepG2 cells.

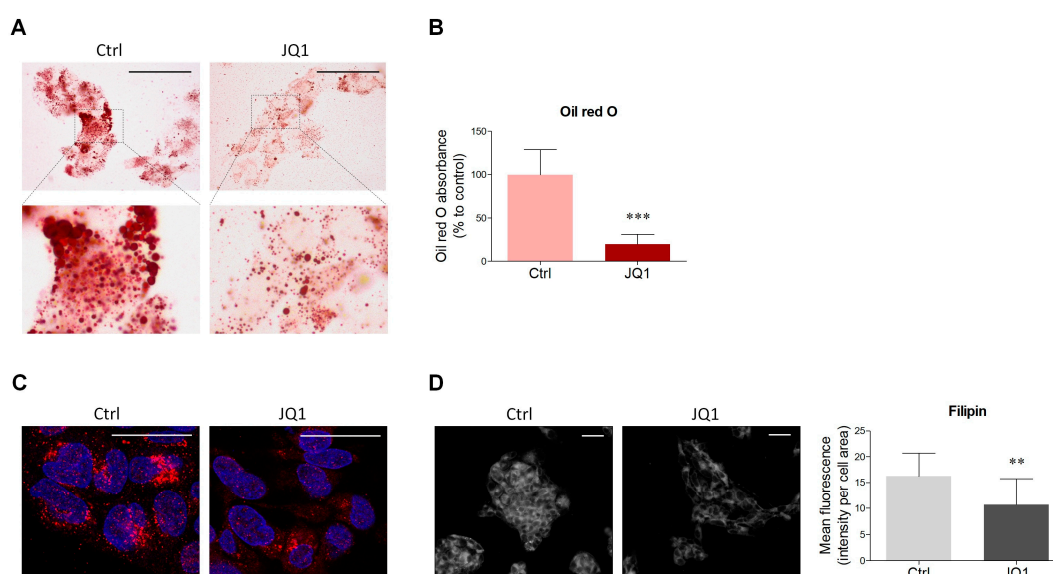


Figure 1. Effects of bromodomain and extraterminal domain (BET) inhibition by JQ1 on intracellular lipids in HepG2 cells. **(A)** HepG2 cells were treated with vehicle (Ctrl) or JQ1 (0.4 μ M), and after 48 hours they were stained with Oil Red O as described in the Materials and Methods Section to visualize the intracellular content of neutral lipids. $n = 6$ different experiments. Scale bar: 50 μ m **(B)** HepG2 cells were treated as in (A), and Oil Red O was extracted with isopropanol. The eluted dye was then quantified by spectrophotometry to evaluate the amount of neutral lipids. $n = 6$ different experiments. **(C)** Vehicle- and JQ1-treated HepG2 cells were fixed and stained with antibody against Plin2 (red). DAPI was used as a nuclear counterstain. Scale bar: 25 μ m **(D)** Representative image (left panel) and quantification of the mean fluorescence intensity (right panel) of filipin staining performed on HepG2 cells treated with vehicle and JQ1 for 48 hours. $n = 5$ different experiments. Scale bar: 50 μ m. Data represent means $\pm$ SD. Statistical analysis was performed by using unpaired Student's t test. ** $p < 0.01$; *** $p < 0.001$.

2.2. BET Inhibition by JQ1 Modulates the Expression of Proteins and Enzymes Involved in Lipid Metabolism

To understand the cellular mechanisms underlying the reduction of lipid content induced by BET inhibition, the prospective modulation of proteins belonging to the lipid metabolism machinery were assessed. The analysis was initially focused on ACC and HMGCR, the rate-limiting enzymes involved in fatty acid and cholesterol biosynthesis, respectively. Western blot analysis revealed that JQ1 treatment significantly decreased ACC protein expression if compared to vehicle-treated HepG2 cells (Figure 2A). However, no changes were observed in the ratio between the phosphorylated fraction of ACC and its total levels, suggesting that BET inhibition modulated the protein amount of the enzyme without influencing its activation state by inhibitory phosphorylation. Similar results were obtained by analyzing HMGCR; in fact, JQ1 administration strongly reduced the protein levels of the enzyme without affecting its phosphorylation state (Figure 2B). The effect of BET inhibition on

HMGCR expression was further confirmed by confocal analysis, showing an overall decrease of immunofluorescence intensity in JQ1-treated HepG2 with respect to control cells (Figure 2C).

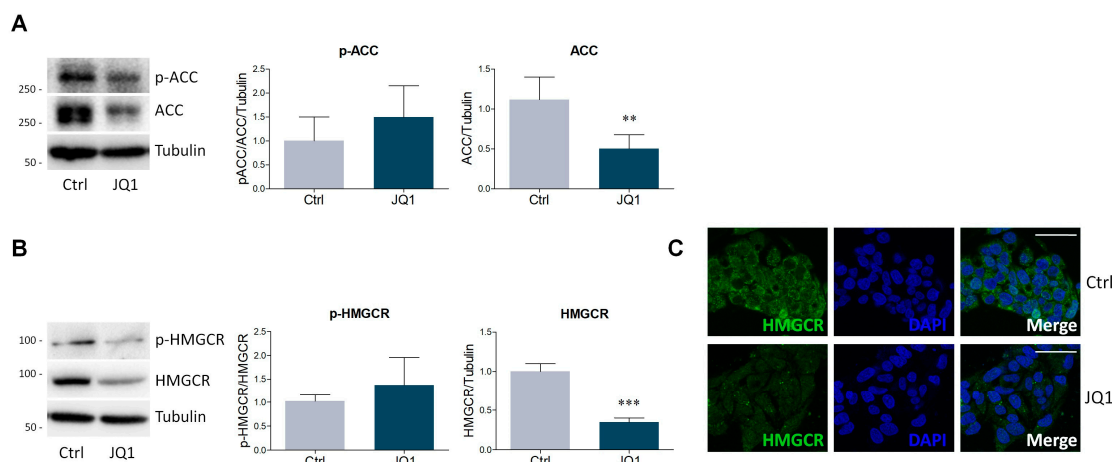


Figure 2. Evaluation of BET inhibition on lipid biosynthesis enzymes. **(A)** Representative Western blot (left panel) and densitometric analysis of phosphorylated Acyl Coenzyme A carboxylase (ACC) (P-ACC, ser79) and total ACC in HepG2 cells treated with vehicle (Ctrl) or JQ1 (0.4 μ M) for 48 hours. $n = 6$ independent experiments. Tubulin was employed as a housekeeping protein to normalize protein loading. **(B)** Representative Western blot (left panel) and densitometric analysis (right panel) of phosphorylated 3-hydroxy-3-methylglutaryl Coenzyme A reductase (HMGCR) (p-HMGCR, ser872) and total HMGCR in HepG2 cells treated with vehicle (Ctrl) or JQ1 (0.4 μ M) for 48 hours. $n = 7$ independent experiments. Tubulin served as a housekeeping protein to normalize protein loading. **(C)** Immunofluorescence staining of HMGCR (green) of HepG2 cells treated as in (B). Nuclei were counterstained with DAPI. $n = 3$ different experiments. Scale bar: 50 μ m. Data are expressed as means $\pm$ SD. Statistical analysis was carried out by using unpaired Student's *t* test. ** $p < 0.01$; *** $p < 0.001$.

Lipid homeostasis is guaranteed by a delicate equilibrium between biosynthesis and extracellular uptake. The latter process is mainly operated by LDLr, which internalizes LDL through receptor-mediated endocytosis [32]. In addition to LDLr, hepatic cells also express SR-B1, a multiligand receptor that binds several lipoproteins, including HDL and LDL [33]. Considering their pivotal role in the physiological regulation of lipid metabolism, the prospective effects mediated by BET inhibition were also assessed for these two lipoprotein receptors. SR-B1 expression was significantly repressed by JQ1 treatment, as observed by Western blot and immunofluorescence data (Figure 3A,B). Similarly, BET inhibition determined a three-fold reduction in LDLr expression levels (Figure 3C). Immunofluorescence microscopy confirmed this result, being LDLr barely detectable in JQ1-treated HepG2 cells when compared to vehicle-treated cells (Figure 3D).

Upon binding to lipoprotein receptors, LDL are internalized and transported throughout the endocytic pathway to lysosomes, where cholesteryl esters can be hydrolyzed by acid lipases [34]. Subsequently, unesterified cholesterol exits the lysosomal compartment, through the activity of NPC1, and is delivered to the plasma membrane and the endoplasmic reticulum (ER) [35]. Because of its essential role in intracellular cholesterol trafficking, Niemann-Pick type C1 (NPC1) protein levels were assessed in this study. BET inhibition by JQ1 strongly enhances NPC1 protein expression in HepG2 cells (Figure 3E). Interestingly, the rise in NPC1 levels was accompanied by a significant reduction of transmembrane protein 97 (TMEM97) protein content, also known as the sigma-2 receptor (Figure 3F), which has already been involved in NPC1 regulation [36].

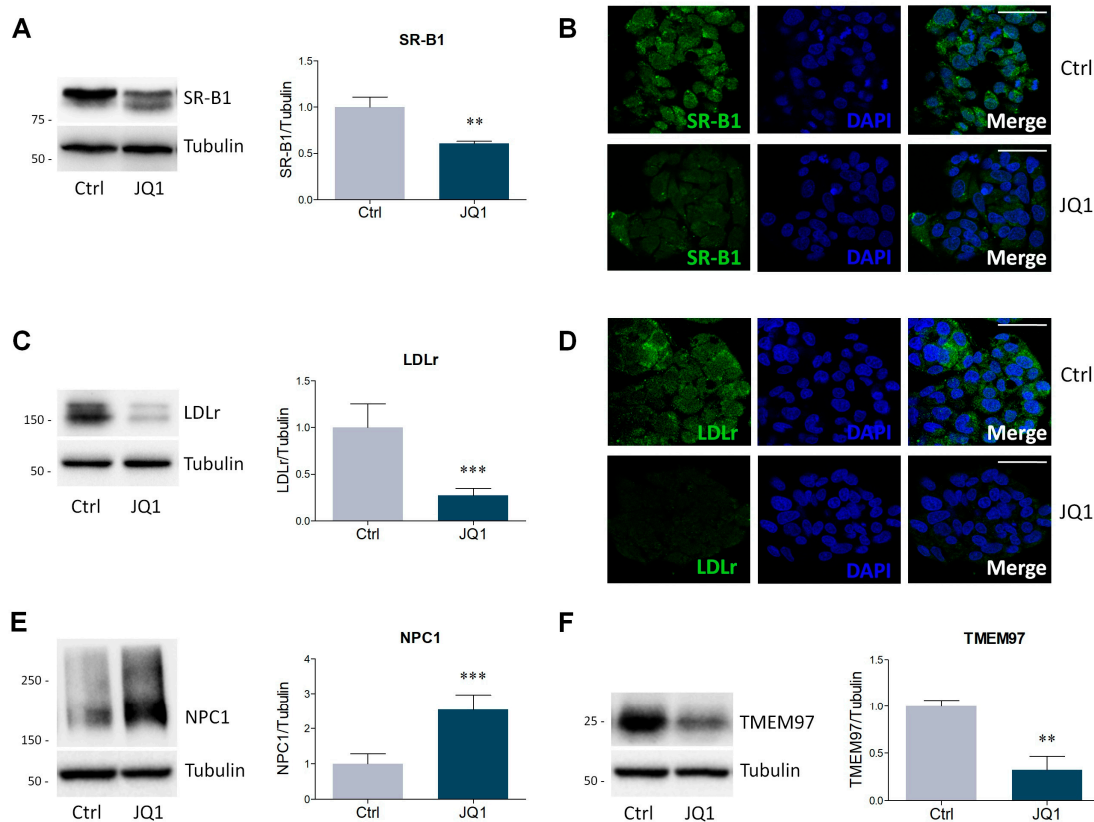


Figure 3. Expression of proteins involved in extracellular lipid uptake and intracellular cholesterol trafficking following JQ1 (0.4 μ M) administration to HepG2 cells for 48 hours. **(A)** Representative Western blot (left panel) and densitometric analysis (right panel) of SR-B1. $n = 6$ independent experiments. **(B)** Immunofluorescence analysis of SR-B1 (green). Nuclei were counterstained with DAPI. $n = 3$ different experiments. Scale bar: 50 μ m. **(C)** Representative Western blot (left panel) and densitometric analysis (right panel) of LDLr. $n = 5$ independent experiments. **(D)** LDLr immunofluorescence (green). Nuclei were counterstained with DAPI. $n = 3$ different experiments. **(E–F)** Representative Western blots and densitometric analysis of NPC1 and TMEM97. Tubulin was chosen as loading control. $n = 6$ independent experiments. Data represent means $\pm$ SD. Statistical analysis was performed by using unpaired Student's *t* test. ** $p < 0.01$; *** $p < 0.001$.

Most proteins involved in lipid metabolism are under the transcriptional control of SREBPs. Considering the effects of BET inhibition evaluated in this work, the expression of SREBP-1 and SREBP-2 was estimated. JQ1 treatment induced a significant increase of SREBP-1 precursor (full-length, FL SREBP-1). However, this effect was not paralleled by a concurrent modification in the nuclear and transcriptionally active fragment of SREBP-1 (nSREBP-1), as its expression is similar between the two experimental groups (Figure 4A). Morphological analysis corroborated this evidence, revealing a slightly higher fluorescence intensity in the cytosolic compartment in JQ1-treated cells, consistent with the increase of FL SREBP-1 observed by the Western blot. On the contrary, no differences were detected in the number and intensity of the stained nuclei, reflecting the lack of significant alterations in the amount of nSREBP-1 (Figure 4B). Differently from SREBP-1, BET inhibition reduced the expression of both precursor (FL SREBP-2) and nuclear SREBP-2 (nSREBP-2) (Figure 4C). SREBP-2 immunofluorescence revealed a predominant nuclear staining, and a weak signal in the cytoplasmic compartment. Notably, JQ1 administration markedly decreased the intensity and the number of nuclei stained for SREBP-2 (Figure 4D).

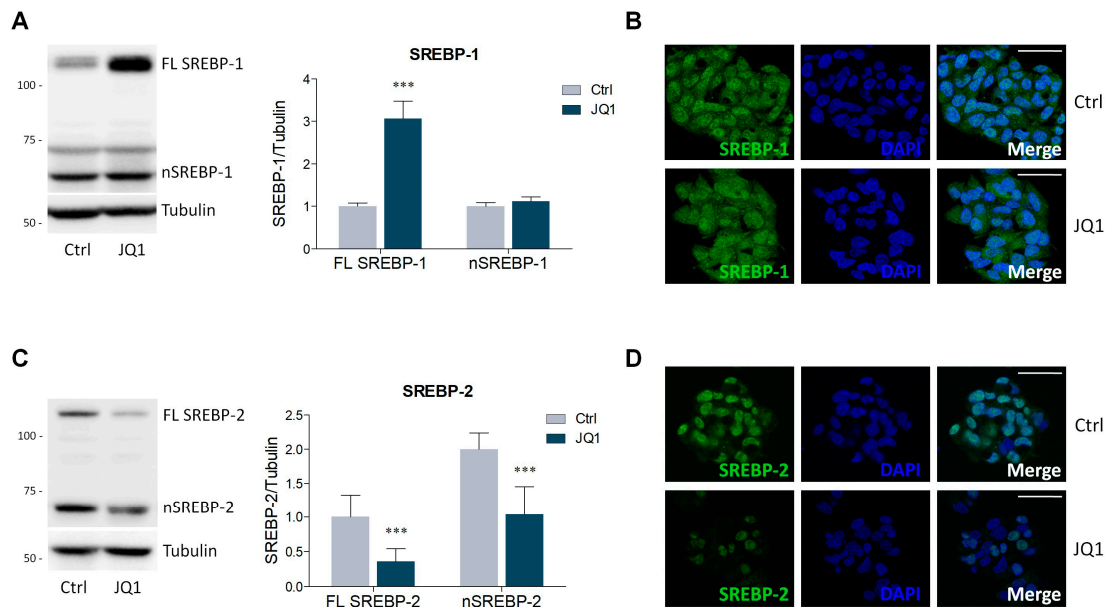


Figure 4. BET inhibition alters sterol regulatory element binding proteins (SREBPs) expression in HepG2 cells treated with JQ1 (0.4 μ M) for 48 hours. **(A)** Representative Western blot (left panel) and densitometric analysis (right panel) of SREBP-1. FL SREBP-1 (Full-length SREBP-1); nSREBP-1 (nuclear SREBP-1). $n = 6$ independent experiments. Tubulin was employed for control loading. **(B)** SREBP-1 immunofluorescence staining (green) in HepG2 cells. Nuclei were counterstained with DAPI. $n = 3$ different experiments. Scale bar: 50 μ m. **(C)** Representative Western blot (left panel) and densitometric analysis (right panel) of SREBP-2. FL SREBP-2 (Full-length SREBP-2); nSREBP-2 (nuclear SREBP-2). $n = 6$ independent experiments. Tubulin was used as a housekeeping protein. **(D)** Immunofluorescence analysis of SREBP-2 (green). Nuclei were counterstained with DAPI. $n = 3$ different experiments. Scale bar: 50 μ m. Data represent means $\pm$ SD. Statistical analysis was assessed by using unpaired Student's t test. *** $p < 0.001$.

To further strengthen the effects induced by JQ1 on lipid homeostasis in HepG2 cells, we recapitulated the most relevant findings in different cell lines. As expected, BET blockade significantly suppressed the expression of SREBP-2 and of its targets LDLr and HMGCR also in the neuroblastoma cell line N1E-115 (Figure 5A,B) and in primary culture of human fibroblasts (Figure 5C,D).

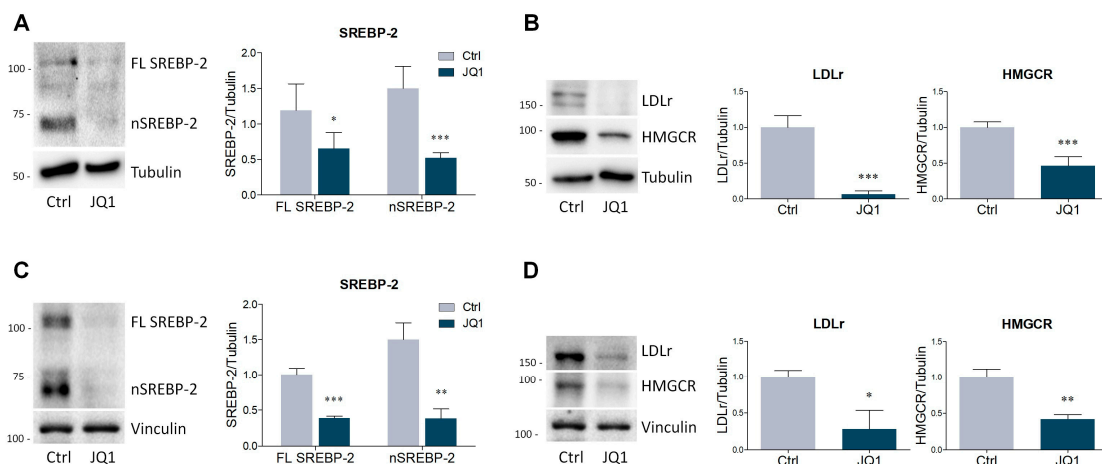


Figure 5. BET inhibition modulates the expression of SREBP-2, HMGCR and LDLr in different cell lines. **(A–B)** Representative Western blot and densitometric analysis of SREBP-2, HMGCR and LDLr in differentiated N1E-115 treated with JQ1 (0.1 μ M) for 48 hours. **(C–D)** Representative Western blot and densitometric analysis of SREBP-2, HMGCR and LDLr in primary human fibroblasts treated with JQ1 (0.4 μ M) for 48 hours. $n = 3$ different experiments. Tubulin and vinculin were used as loading control. Data represent means $\pm$ SD. Statistical analysis was assessed by using unpaired Student's t test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Overall, these data indicate that BET inhibition deeply affects the main proteins involved in lipid biosynthesis, uptake and intracellular transport.

2.3. BET Inhibition Affects Cell Proliferation in a Cholesterol-Dependent Manner

The maintenance of a proper amount of lipids, and in particular of cholesterol, is crucial for several biological processes, such as cell growth and cell proliferation [4,37]. In addition, it has been extensively demonstrated that BET inhibition promotes the suppression of cell proliferation in a number of normal and cancer cell types [19,38,39]. Results collected in this work are in agreement with previous reports, demonstrating that JQ1 treatment significantly slowed down the proliferation rate of HepG2 cells starting at 48 hours from the pharmacological treatment (Figure 6A). Remarkably, the cell growth rate was rescued when JQ1 was co-administered with mevalonate (MVA), the product of the reaction catalyzed by HMGCR. The involvement of cholesterol biosynthesis in the anti-proliferative effects mediated by BET inhibition was further supported by the co-administration of cholesterol to JQ1-treated cells that, similarly to MVA addition, was able to restore cell proliferation. In order to delve deeper into the effects exerted by BET inhibition on cell proliferation and cholesterol modulation, we generated a HepG2 lineage with acquired resistance to JQ1 (HepG2-R) by continuously treating cells with increasing doses of the drug. While JQ1 decreased the number of JQ1-sensitive cells as previously shown, no effects were induced in HepG2-R upon drug administration (Figure 6B). Importantly, the restoration of cell proliferation observed in HepG2-R cells was completely abolished by 25-hydroxycholesterol (25OHC) treatment, a well-known methodological approach to mediate HMGCR degradation through the activation of a feedback mechanism [40]. Similar results were also obtained by blocking HMGCR activity with simvastatin, a potent inhibitor of cholesterol biosynthesis (Figure 6C). Because statins suppress the production of isoprenoid intermediates in the cholesterol biosynthetic pathway, they can exert a plethora of pleiotropic actions independently from cholesterol decrease [8]. To confirm a direct involvement, cholesterol was then administered to HepG2-R cells co-treated with JQ1 and simvastatin. Cholesterol supplementation efficiently prevented the reduction of cell proliferation induced by simvastatin in HepG2-R cells, thus restoring the acquired resistance to JQ1 as a function of cell growth (Figure 6D).

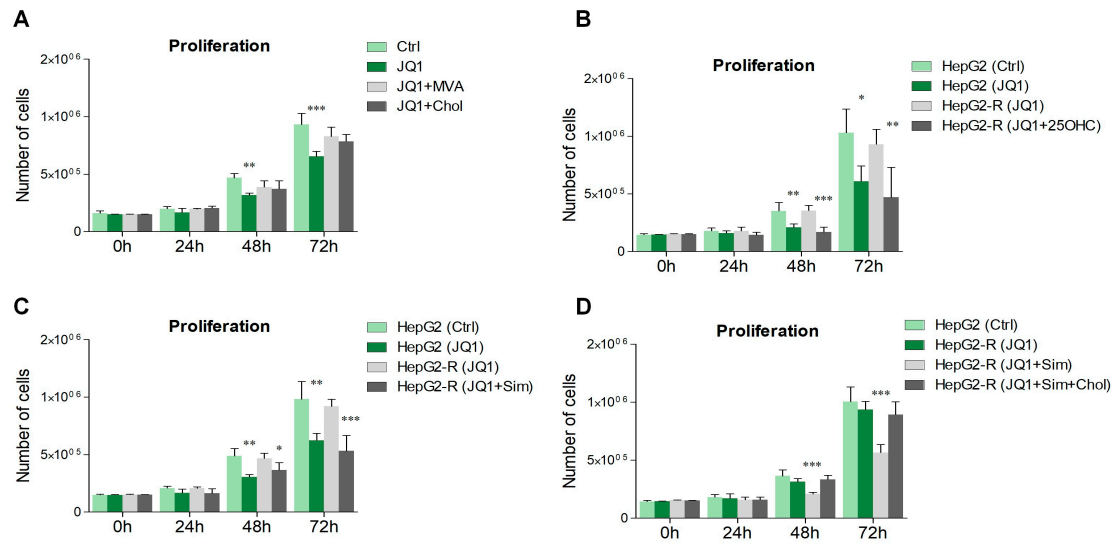


Figure 6. BET inhibition influences cell proliferation through cholesterol metabolism. **(A)** HepG2 cells were seeded in 6-well plates (150,000 cells for each well) and were treated with JQ1 (0.4 μ M), mevalonate (MVA, 100 μ M) and cholesterol (chol, 50 μ M) for 72 hours. Cell counts were conducted over time with a hemocytometer. **(B–C)** Cell proliferation was evaluated in JQ1-sensitive (HepG2) and JQ1-resistant (HepG2-R) cells. JQ1-resistant and -sensitive HepG2 cells were treated with the BET inhibitor for 72 hours, and additional groups of HepG2-R cells were co-stimulated with JQ1+25-hydroxycholesterol (25OHC, 20 μ M) or JQ1+simvastatin (Sim, 1 μ M). Other cells were treated with vehicle and served as control (Ctrl). **(D)** Cell proliferation was evaluated in JQ1-sensitive (HepG2) and JQ1-resistant (HepG2-R) cells. JQ1-resistant cells were constantly stimulated with the BET inhibitor. Additional groups of HepG2-R cells were co-stimulated with JQ1+simvastatin (Sim, 1 μ M) or JQ1+Sim+cholesterol (Chol, 50 μ M). $n = 4$ independent experiments. Data represent means $\pm$ SD. Statistical analysis was assessed by using one-way ANOVA, followed by Dunnett's post hoc. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Overall, these results suggested that the anti-proliferative effects elicited by JQ1 can be mediated by the suppression of cholesterol metabolism and that the acquisition of resistance to BET inhibition may be accompanied by adaptive changes in the main proteins controlling cholesterol homeostasis. Western blot data confirmed this hypothesis, highlighting that both FL SREBP-2 and nSREBP-2 levels were increased in JQ1-resistant HepG2 cells (Figure 7A). Coherently, the expression of SREBP-2 target genes HMGCR, SR-B1 and LDLr was properly restored at the level of control cells in HepG2-R cells (Figure 7B–D). Taken together, these results suggest that the impact of HepG2 proliferation mediated by BET inhibition is dependent on cholesterol metabolism.

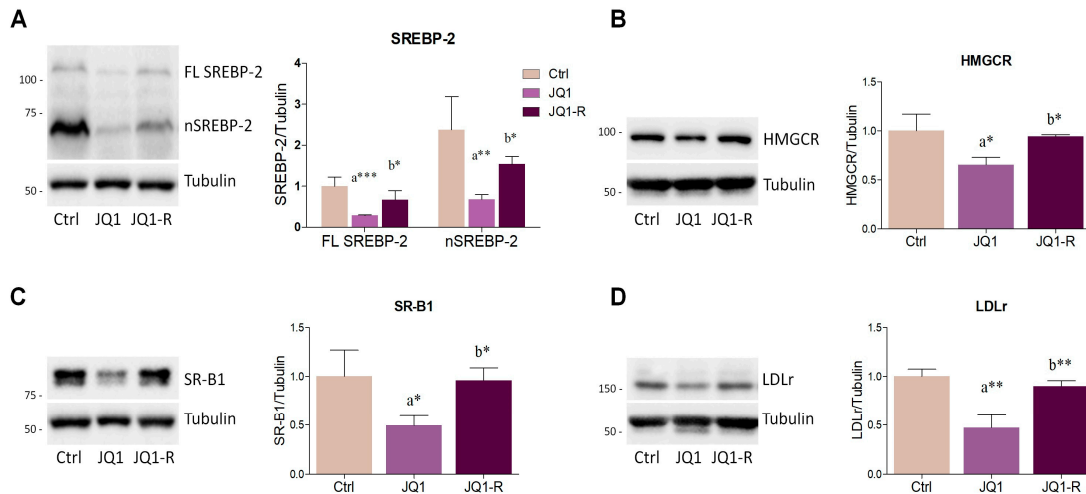


Figure 7. JQ1 resistance is accompanied by the upregulation of proteins controlling cholesterol metabolism. (A–D) Representative Western blots and densitometric analysis of SREBP-2, HMGCR, SR-B1 and LDLr in JQ1-sensitive cells treated with vehicle (Ctrl) or JQ1 (0.4 μM), and in HepG2-resistant (JQ1-R) cells constantly stimulated with JQ1 (0.4 μM). Experiments were performed after 48 hours. $n = 4$ independent experiments. Tubulin served as loading control. Data represent means $\pm$ SD. Statistical analysis was assessed by using one-way ANOVA followed by Tukey's post-hoc. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. "a" indicates statistical significance versus control group (Ctrl); "b" indicates statistical significance compared to JQ1 group.

3. Discussion

The homeostatic maintenance of lipid metabolism plays a fundamental role in assuring the correct development of cellular functions. As a consequence, alterations in the mechanisms controlling lipid balance may be associated to several pathological conditions, ranging from cardiovascular diseases and lipidosis to neurodegenerative and neurodevelopmental diseases [13–15,41]. The discovery of effective lipid-lowering medications, such as statins and fibrates, have revolutionized the treatment and the clinical outcomes of different lipid disorders [42]. Unfortunately, numerous diseases associated to lipid disturbances do not yet have a resolutive therapy. The obstacles in designing efficient pharmacological approaches relies on the fact that cellular and molecular mechanisms regulating lipid metabolism are not completely elucidated.

In this context, it is important to delve deeper into the regulatory mechanisms of lipid homeostasis, with the attempt to unravel potential etiopathological factors and novel molecular targets that could be useful to set up promising therapies. In the last few years, several reports highlighted that epigenetic factors may play crucial roles in the control of lipid homeostasis. For instance, it is becoming increasingly clear that histone deacetylases (HDAC) and microRNAs exert a crucial modulatory activity in lipid and energy metabolism [43–47]. On the contrary, the involvement of BET proteins in the regulation of lipid homeostasis is still elusive. Thus, in this work, we evaluated the effects of BET inhibition in the expression of the main proteins controlling lipid metabolism.

Collectively, our results demonstrated that BET inhibition induced an overall suppression of lipid metabolism. In particular, JQ1 administration decreased the intracellular lipid content and reduced the expression of biosynthetic enzymes (ACC and HMGCR) as well as of receptors involved in lipoprotein uptake (LDLr and SR-B1). BET inhibition also induced a strong downregulation in the expression of TMEM97. This conserved integral membrane protein has been identified as an important modulator of cholesterol levels and, similarly to other proteins coordinating lipid metabolism, is a SREBP-2 target gene [36,48,49]. It is well described that SREBPs isoforms possess different roles in lipid biosynthesis. SREBP-1 isoforms are mostly devoted to the regulation of fatty acid and triglyceride metabolism, whereas SREBP-2 is relatively selective in activating cholesterol-

related genes [50,51]. Coherently with these notions, the JQ1-mediated suppression of HMGCR, LDLr, SR-B1 and TMEM97 was paralleled by a concurrent decrease of both the full-length and the nuclear active fraction of SREBP-2. Interestingly, BET inhibition was also responsible for a significant decrease of ACC expression, which, however, was not adequately accompanied by changes in nSREBP-1 levels. Indeed, the levels of SREBP-1 precursor were significantly augmented, probably representing a transcriptional attempt to counteract the marked decrease of the SREBP-2 isoform. Conversely, the expression of the nuclear and transcriptionally active fragment of SREBP-1 was unaltered upon JQ1 treatment, excluding its involvement in the modulation of ACC levels. Even though ACC transcription is preferentially controlled by SREBP-1 isoforms [50], it has been observed that SREBP-2 can equally influence ACC transcription [52], supporting the notion that the reduction of ACC levels observed in this work may be ascribable to the suppression of SREBP-2 mediated by JQ1. In addition, BET blockade led to a rise in NPC-1 protein levels. Literature data illustrated that a reduction of TMEM97 increases NPC-1 protein expression by a post-translational mechanism [36], and suggest that the suppression of TMEM97 could explain the build-up of NPC-1 observed in this study following JQ1 administration. Consistent with JQ1-mediated decrease of LDLr expression, NPC1 induction may also represent a refined compensatory response to contrast the prospective decrease of LDL-cholesterol uptake. Furthermore, it cannot be excluded that BET inhibition directly affects the transcription of *NPC1*, as well as of other genes involved in the maintenance of lipid homeostasis.

Subsequently, the prospective contribution of lipid metabolism in the anti-proliferative effects induced by JQ1 administration was evaluated. It has been extensively reported that BET inhibition exerts a remarkable reduction in cell proliferation by hindering the transcription of oncogenes such as c-Myc [53], and by suppressing the activation of pro-survival and growth signalling kinases like Akt [26]. In this study, we highlight for the first time a novel mechanism by which BET inhibition modulates cell proliferation in a cholesterol-dependent manner. Indeed, the administration of both MVA and cholesterol to culture medium efficiently abolished the delay in cell proliferation induced by JQ1. Consistently, the generation of HepG2-R cells further corroborated this evidence, as the acquisition of resistance to JQ1 is associated to the compensatory increase in the expression of proteins belonging to cholesterol metabolism, and to the restoration of cell proliferation rate.

Overall, these data suggest that BET inhibition reduces lipid content by hampering SREBP-2 expression and processing, which result in a reduced expression of target genes involved in lipid biosynthesis, uptake and intracellular trafficking.

Despite the fact that more efforts should be done in order to better clarify the specific contribution of each BET protein in the regulation of lipid metabolism, this work provides the proof of principle that epigenetic pathways, influenced by BET protein activity, represent novel physiological mechanisms to control lipid homeostasis. These findings may also set the basis for designing innovative therapeutic approaches aimed at ameliorating the functional outcomes of several disorders characterized by lipid unbalances.

4. Materials and Methods

4.1. Cell Cultures and Generation of HepG2 Resistant to JQ1

N1E-115 neuroblastoma cells were cultured at 5% CO₂ in DMEM medium at high glucose, containing 10% (*v/v*) foetal calf serum, L -glutamine (2 mM), and added with penicillin/streptomycin solution. Cells were then seeded at 50% confluency and were induced to differentiate for four hours by adding 2% dimethyl sulfoxide. Differentiated N1E-115 cells were treated with JQ1 (0.1 µM) or vehicle (DMSO, dilution 1:1000 in cell culture media) for 48 hours.

Human foetal foreskin fibroblasts (HFFF2) were grown at 5% CO₂ in DMEM (high glucose) additioned with 10% foetal calf serum, L -glutamine (2 mM), and penicillin/streptomycin solution. HFFF2 (60% confluency) were then treated with 0.4 µM JQ1 for 48 hours. Cells treated with vehicle (DMSO, dilution 1:1000 in cell culture media) served as control.

HepG2 cells were routinely cultured at 5% CO₂ in DMEM medium at high glucose, containing 10% (*v/v*) foetal calf serum, L -glutamine (2 mM), and added with penicillin/streptomycin solution. Cells were passaged every 3 days and medium changed every 2–3 days. All the experiments were performed with cell confluency of 60%–70%. DMSO stock of 4 mM JQ1 was diluted 1:1000 in cell culture media to obtain the final concentration of 0.4 μ M JQ1. Experiments were then performed 48 hours after JQ1 stimulation. Cells treated with vehicle (DMSO, dilution 1:1000 in cell culture media) served as control. The generation of HepG2 cells resistant to JQ1 was carried out by slightly modifying the protocol provided by Gobbi and colleagues [54]. HepG2 resistant cells were selected by continuously applying increasing doses of JQ1, starting from 50 nM and enhancing the drug concentration every 1–2 weeks for 2 months of total treatment. Surviving JQ1-resistant cells were then maintained at 0.4 μ M JQ1 throughout. For cell proliferation experiments, chemicals were purchased from Sigma-Aldrich and used at the following concentrations: JQ1 (0.4 μ M), mevalonate (100 μ M), cholesterol (50 μ M), simvastatin (1 μ M), 25-hydroxycholesterol (20 μ M).

4.2. Oil Red O Staining and Quantification

For the evaluation of lipid content, HepG2 cells were seeded in 12-well plates. Briefly, after 48 hours of JQ1 treatment, cells were fixed in paraformaldehyde (4% solution) for 10 minutes and gently rinsed twice with PBS. Sixty per cent isopropanol was added to fixed cells for 5 minutes, and then washed 2 times with distilled water. Cells were stained with 1 mL of the Oil Red O solution (Sigma-Aldrich, Milan, Italy) for 15 minutes at room temperature with continuous gentle shaking. Subsequently, wells were then rinsed 3 times with distilled water, until no excess stain was seen. Stained cells were visualized in brightfield using an Olympus BX 51 microscope (Olympus Italia, Segrate, Italy), equipped with a Leica DFC 420 camera (Leica Microsystems, Milan, Italy). Electronic images were captured using a Leica Application Suite version 3.5.0 system (Leica Microsystems, Milan, Italy). For relative Oil Red O accumulation by spectrophotometry, after incubation with Oil Red O solution, stained cells were washed 3 times with distilled water, and the dye was eluted by the addition of 1 mL isopropanol for 15 minutes, with gentle shaking. A total of 100 μ L of the eluted dye was removed from each sample and were transferred to a clean 96-well plate for reading the absorbance at 540 nm.

4.3. Filipin Staining

Filipin staining was performed as previously described [55]. Filipin staining was performed using Filipin complex (Sigma-Aldrich, catalog #F9765). Filipin stock solution (10mg/mL in PBS) was freshly prepared before the use. Cells were fixed in paraformaldehyde (4% solution) for 10 minutes and rinsed 3 times with PBS. Cells were stained with 1mL Filipin working solution (0.05 mg/mL in PBS) for 2 hours at room temperature, in the dark. Next, wells were rinsed 3 times with PBS to remove excess dye. Finally, cells were viewed in PBS through a fluorescence microscope using a UV filter set (340–380 nm excitation). Images were acquired at 20 $\times$ magnification using an Axio Imager Z2 (Carl Zeiss, Jena, Germany) equipped with a charge-coupled device (CCD) camera controlled by the ISIS software (MetaSystems, Milano, Italy). Filipin quantification was calculated as the mean fluorescence intensity per cell area by using ImageJ Software for Windows.

4.4. Lysate Preparation and Western Blot Analysis

HepG2 lysate was performed as already reported [56]. Forty-eight hours after treatment, cells were lysed in 80 μ L lysis buffer (0.25 M Tris pH 6.8, 10% SDS, phosphatase and protease inhibitor cocktails) by sonication (duty cycle 20%, output 3). Samples were then centrifuged at 10,000g for 10 min to remove cell debris. Protein concentration was assessed by the method of Lowry. Subsequently, Laemmli buffer was added to HepG2 lysates, and samples were boiled for 3 min before loading to the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for subsequent western blot analysis.

Western blot experiments were performed by slightly modifying the previously described protocol [8]. Briefly, proteins (30 µg) from HepG2 lysates were resolved on SDS-PAGE at 40 mA (constant current) for 60 min. Subsequently, proteins were transferred onto nitrocellulose membrane by using Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Milan, Italy). The nitrocellulose membrane was incubated at room temperature with 5% fat-free milk in Tris-buffered saline (0.138 M NaCl, 0.027 M KCl, 0.025 M Tris-HCl, and 0.05% Tween-20, pH 6.8), and probed at 4 °C overnight with the following primary antibodies: phospho-HMGCR (Merck Millipore, #09-356, dilution 1:1000), HMGCR (Abcam, ab242315, dilution 1:1000), phospho-ACC (Sigma-Aldrich, SAB4503851, dilution 1:500), ACC (Sigma-Aldrich, SAB4501396, dilution 1:500), SR-B1 (Abcam, Cambridge, UK, ab52629, dilution 1:2000), LDLr (Abcam, ab30532, dilution 1:1000), NPC1 (Novus Biologicals, NB400-148, dilution 1:1000), TMEM97 (Novus Biologicals, Centennial, CO, USA, NBP1-30436, dilution 1:1000), SREBP-2 (Abcam, ab30682, dilution 1:1000), SREBP-1 (Santa Cruz Biotechnology, sc-8984, dilution 1:1000), alpha-Tubulin (Sigma-Aldrich, T6199, dilution 1:10000), vinculin (Sigma-Aldrich, V9131, dilution 1:20,000). Subsequently, membranes were probed for 1 hour with horseradish peroxidase conjugated secondary IgG antibodies (Bio-Rad Laboratories, Milan, Italy). Protein-antibody immunocomplexes onto nitrocellulose were visualized by using clarity ECL Western blotting (Bio-Rad Laboratories, Milan, Italy, #1705061), and chemiluminescence acquisition was carried out through ChemiDoc MP system (Bio-Rad Laboratories). Western blotting images were analyzed by ImageJ (National Institutes of Health, Bethesda, MD, USA) software for Windows. All samples were normalized for protein loading with alpha-Tubulin (chosen as a housekeeping protein). Recorded values were derived from the ratio between arbitrary units obtained from the protein band and the respective housekeeping protein.

4.5. Immunofluorescence Staining

Immunofluorescence of HepG2 cells was performed by following the previously described protocol [17]. Cells were fixed in paraformaldehyde (4% in PBS) and incubated overnight with appropriate antibodies: HMGCR (Abcam, ab242315, dilution 1:100), SREBP-2 (Abcam, ab30682, dilution 1:100), SREBP-1 (Santa Cruz Biotechnology, Dallas, TX, USA, sc-8984, dilution 1:100), SR-B1 (Abcam, ab52629, dilution 1:100), LDLr (Santa Cruz Biotechnology, sc-11824, dilution 1:200), anti-Perilipin-2 (anti-Plin2) antibody (R&D Systems, #MAB76341, dilution 1:100). After incubation with primary antibodies, fixed cells were probed for 1 hour at room temperature with donkey anti-goat secondary antibody Alexa Fluor 488 (ThermoFisher Scientific, Milan, Italy, A-11055), goat anti-rabbit secondary antibody Alexa Fluor 555 (ThermoFisher Scientific, A27039) and goat anti-rabbit secondary antibody Alexa Fluor 488 (ThermoFisher Scientific, A-11008). Coverslips were mounted with Vectashield Antifade mounting medium with DAPI (Vector, H-1200) to visualize nuclear staining. The samples were examined at confocal microscopy (TCS SP8; Leica, Wetzlar, Germany). Images were captured using Leica TCS SP8 equipped with a 40 × 1.40–0.60 NA HCX Plan Apo oil BL objective at RT and Leica LAS X Software.

4.6. Statistical Analysis

All the results are expressed as mean ± standard deviation (SD). Normal distribution of the data was assessed by applying the Shapiro–Wilk test. Unpaired Student's *t* test was performed to compare means between two experimental groups. When comparing three or more experimental groups, one-way analysis of variance (ANOVA) was carried out, followed by Tukey's or Dunnett's post hoc. *p* < 0.05 was considered to indicate a statistically significant difference. Statistical analysis and graph editing were performed using GRAPHPAD INSTAT3 (GraphPad, La Jolla, CA, USA) for Windows.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ACC	Acyl Coenzyme A carboxylase
BET	Bromodomain and extra-terminal domain
HDAC	histone deacetylases
HDL	high density lipoprotein
HMGCR	3-hydroxy-3-methylglutaryl Coenzyme A reductase
LDL	low density lipoprotein
LDLr	low density lipoprotein receptor
NPC1	Niemann-Pick type C1
ROS	reactive oxygen species
SR-B1	scavenger receptor class B type 1
SREBPs	sterol regulatory element binding proteins
TMEM97	transmembrane protein 97

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Inhibition of Bromo- and Extra-Terminal domain (BET) proteins promotes neuronal differentiation by inducing cholesterol metabolism rearrangements

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Abstract

Neuronal differentiation is a crucial physiological process involved in the development and plasticity of nervous tissue. It is associated with reduced cell proliferation and synthesis of specific proteins that will result in changes of cellular morphology and function.

Interestingly, this process depends on cholesterol homeostasis.

Recently, experimental data highlighted that epigenetic factors play a crucial role in the regulation of metabolic processes, particularly in lipid metabolism. In this context, the role of Bromodomain and Extra-Terminal domain (BET) proteins still remains vague.

From these premises, we evaluated whether BET proteins could influence neuronal differentiation and whether this crucial process could be related to a modulation of cholesterol pathway. To reach this goal, we employed a loss-of-function approach by using JQ1, a potent and specific BET inhibitor. Our results demonstrated that BET inhibition by JQ1 induces neuritogenesis in different cell culture models of neuronal differentiation. Notably, this effect is accompanied by a significant modulation of the main proteins and enzymes involved in cholesterol metabolism. Furthermore, we demonstrated that JQ1-mediated pro-differentiating effects are also associated with the activation of specific signaling cascades and of autophagy, which are a prerequisite to assure proper cholesterol homeostasis during neuritogenesis. In addition, we provided evidence that BET blockade significantly increases the number of Doublecortin-expressing neurons in both the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampal dentate gyrus, suggesting enhanced adult neurogenesis in mice.

These results demonstrate that BET inhibition is a critical epigenetic modulation to control neuronal differentiation. Furthermore, the effect of BET inhibitors provides the rationale for

designing novel therapeutic approaches aimed at counteracting neuropathological conditions characterized by altered neurogenesis.

1. Introduction

The central nervous system (CNS) is one of the most complex systems of the body. The stages that determine its development are different and require the intervention of numerous molecular actors. Neuronal differentiation is of paramount importance for brain development. This crucial physiological process is characterized by reduced cell growth and synthesis of specific proteins that will sustain new cellular morphology and function (*Zogovic et al., 2015*).

Neuronal differentiation is essential for neurogenesis, the process that conducts to the formation of new nerve cells from neural stem cells (NSC). Although neurogenesis plays a central role in neurodevelopment, it also occurs in few and well-localized areas of the adult brain (*Gotz and Huttnner, 2005*). There are two main regions where adult NSC maintain proliferative capacity and, following proper stimulation, undergo differentiation programs in glia cells and neurons: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus, but also other sites such as the neocortex, striatum and hypothalamus (*Feliciano et al., 2015; Inta et al., 2015*). The discovery of stem cell niches in the adult mammalian CNS and the evidence regarding the ability of NSC to self-renew and differentiate into mature neurons could provide important therapeutic strategies for the treatment of neurodevelopmental and neurodegenerative diseases.

Various evidence illustrated how cell metabolism controls NSC activity and the subsequent neuronal differentiation (*Knobloch and Jessberger, 2017*). In particular, lipid metabolism was found to be significantly involved in cell proliferation, migration and differentiation, opening new scenarios to the mechanisms that regulate NSC activity. Among the lipids, cholesterol performs fundamental functions in neuron physiology. For instance, it has been demonstrated that cholesterol addition to the culture medium enhances the neuronal differentiation and maturation of SH-SY5Y cells (*Teppola et al., 2016*). The impact of cholesterol on neuronal differentiation is further confirmed by other reports, showing that cholesterol biosynthesis and uptake is increased during differentiation of cortical neurons (*Genaro-Mattos et al., 2019*). Accordingly, derangements in cholesterol metabolism heavily impact on neuronal differentiation of NSC in several pathological conditions, such as Niemann-Pick type C disease (NPC) (*Kim et al., 2007*). Other findings highlight that cholesterol is also essential for synaptogenesis, as it increases the

number of synapses improving their stability (*Suzuki et al., 2007*). Specifically, synaptic vesicle membranes possess a relevant amount of cholesterol compared to other intracellular organelles (*Deutsch and Kelly, 1981*), supporting the hypothesis that synaptic vesicle biogenesis requires elevated cholesterol levels (*Pfriege, 2003*).

Chromatin modifications plays important roles in neurogenesis, driving the expression of multiple genes involved in self-renewal and differentiation (*Nieto-Estevez et al., 2022*). In this context, the role of histone deacetylases (HDAC) is well-established, and different HDAC inhibitors have been proposed as novel therapeutic approaches to treat neurodevelopmental and neurodegenerative disorders (*Shukla and Tekwani, 2020*). Differently, the role of Bromodomain and Extra-Terminal domain (BET) proteins is elusive. BET proteins, namely BRD2, BRD3 and BRD4, are epigenetic readers recruited to the chromatin through the binding to the acetylated histones, thus regulating gene expression (*Wang et al., 2015*). The involvement of this class of epigenetic regulators has been extensively characterized in different types of cancer and inflammatory conditions, due to their role in the transcriptional modulation of oncogenes and mediators of the immune response (*Segatto et al., 2017; Andrieu et al., 2018*). Recently, several studies illustrated that the influence of BET proteins can be extended to the regulation of metabolic processes, including cholesterol homeostasis. For instance, cholesterol biosynthesis is induced by the recruitment of BET proteins to the promoters of several genes coding for proteins belonging to the mevalonate (MVA) pathway and, consistently, overexpression of these epigenetic regulators enhances cholesterol biosynthesis (*Zhang et al., 2017*). Additionally, Western blot and immunofluorescence analysis in different cell lines revealed that JQ1, a powerful and selective BET inhibitor, modulates the expression of proteins belonging to the regulatory network of cholesterol homeostasis (*Tonini et al., 2020*).

As already reported, neuronal differentiation strongly relies on cholesterol metabolism. Thus, we hypothesized that BET proteins may promote neuronal differentiation by modulating cholesterol homeostasis. To reach this aim, a loss-of-function approach was used, and BET proteins were inhibited by the administration of JQ1. The effects were mainly assessed in N1E-115, a murine neuroblastoma cell line, widely used in the study of neuronal differentiation (*Kranenburg et al., 1995*). Subsequently, the impact of BET inhibition was further assessed in isolated mouse NSC and *in vivo* during adult neurogenesis.

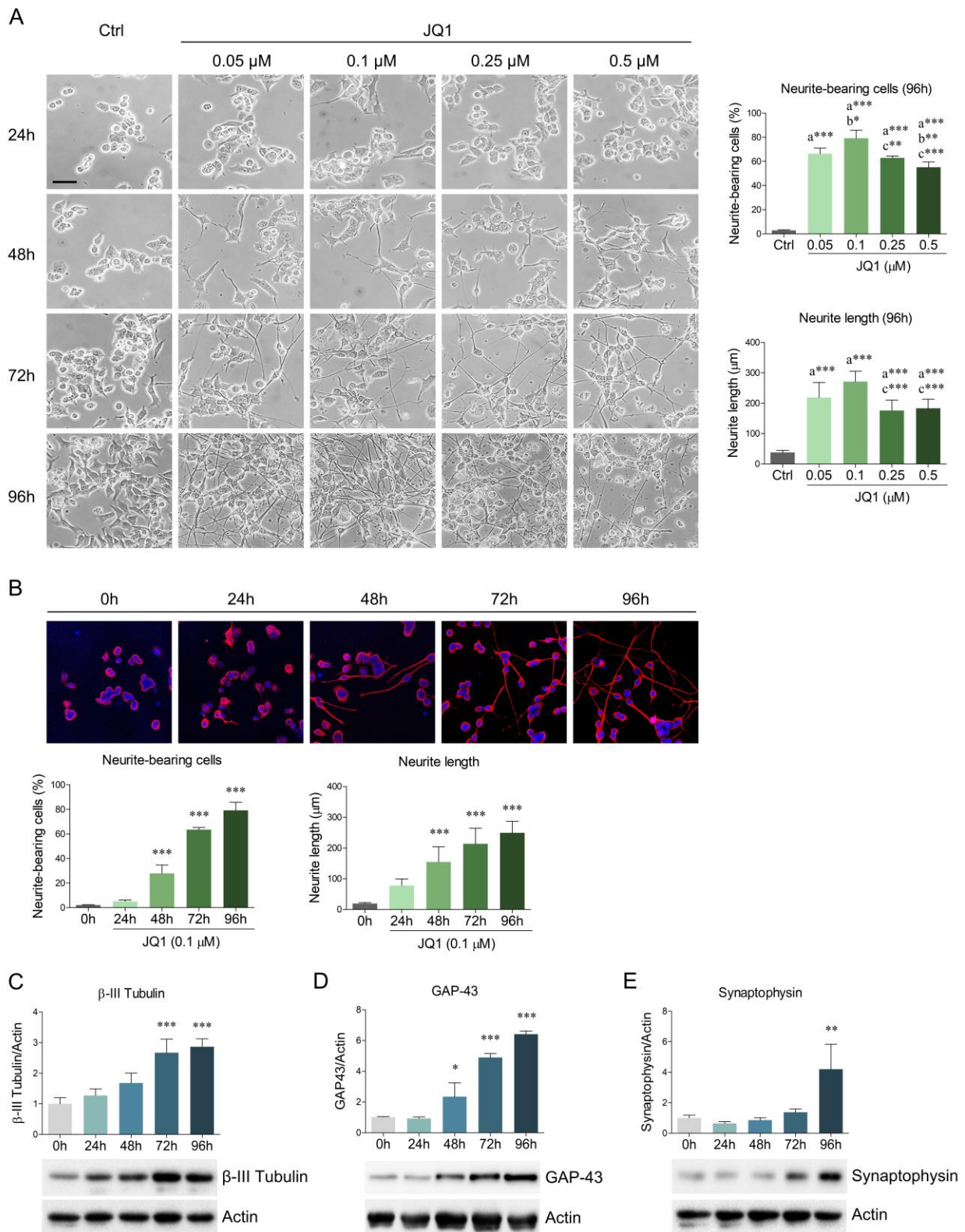
2. Results

2.1 BET inhibition by JQ1 induces neuronal differentiation in N1E-115 cells

In order to evaluate the effects of BET inhibition on neuronal differentiation, a morphological analysis of N1E-115 cells treated with different doses of JQ1, up to 96 hours, was performed. Figure 1A (left panel) indicates that JQ1 is a strong inducer of neuronal differentiation. Cells treated with JQ1 show extensive neurite formation starting from 48 hours, unlike the respective controls which instead maintain a rounded shape with few short projections. Quantitative analyzes were carried out at terminal differentiation (96 h) by evaluating the percentage of neurite-bearing cells and neurite length (Figure 1A, right panel): JQ1 induces an appreciable neuronal differentiation, which is particularly prominent at a dose of 0.1 μ M.

Subsequently, the differentiation induced by 0.1 μ M of JQ1 was characterized more in detail. The differentiation was evaluated through the confocal analysis of β -III Tubulin, a marker frequently used to assess neuronal differentiation since its expression levels tend to increase during neuritogenesis (*Sieber-Blum et al., 2006*). N1E-115 initially appear undifferentiated (0h) and β -III Tubulin immunostaining is weak. During the time course, immunopositivity becomes intense. Coherently, the number neurite-bearing-cells and the length of neurites progressively increase over time (Figure 1B).

The extent of neuronal differentiation was not only evaluated from a morphological point of view, but also through Western blot analysis of differentiation markers. β -III Tubulin protein levels progressively rises during JQ1-induced differentiation, being significant at 72 and 96 hours (Figure 1C). The protein levels of Growth Associated Protein 43 (GAP-43) and Synaptophysin were also analyzed. GAP-43 is expressed at high levels in neuronal growth cones during development and axonal regeneration, representing a crucial component of axons and presynaptic terminals (*Roskothen-Kuhl and Illing, 2014*). Synaptophysin is a protein expressed in almost all neurons and is one of the main proteins involved in the functioning of synaptic vesicles (*Calhoun et al., 1996*). As shown in Figure 1D,E, the expression of GAP-43 and Synaptophysin increases during differentiation, especially at later stages when the cells acquire the features of mature neurons (Figure 1D,E).



one-way ANOVA, followed by Tukey's or Dunnet's post hoc. "a" indicates statistical significance vs Ctrl; "b" indicates statistical significance vs JQ1 0.05 μ M; "c" indicates statistical significance vs JQ1 0.1 μ M. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

2.2 Neuronal differentiation mediated by BET inhibition is associated to arrangements in cholesterol metabolism

Neuronal differentiation leads to a series of metabolic changes affecting cholesterol metabolism (Cartocci *et al.*, 2016). Thus, we assessed whether neurite outgrowth mediated by BET blockade could be correlated to a modulation of cholesterol metabolism. To reach this objective, the transcription and expression levels of the main proteins and enzymes involved in cholesterol metabolism were assessed. To start, we evaluated sterol regulatory element binding protein 2 (SREBP-2), the main transcription factor involved in the transcription of cholesterologenic genes, at both mRNA and protein level. *Srebf2* transcript levels gradually increase during JQ1-induced differentiation. In order to ensure the exact amount of cholesterol the cells need for their physiological function, SREBP-2 undergoes a proteolytic cleavage, leading to the formation of an active fragment (nSREBP-2) that enters the nucleus and promotes the transcription of genes involved in cholesterol biosynthesis and uptake (Brown *et al.*, 2018). In this context, we noticed that the expression of both precursor (FL SREBP-2) and nuclear (n-SREBP2) SREBP-2 significantly increases over time upon JQ1 administration (Figure 2A). We then examined the expression of different SREBP-2 transcriptional targets, at both mRNA and protein level. The attention was initially focused on low-density lipoprotein receptor (LDLr) and LDLr-related protein 1 (LRP1), that are mainly responsible for cholesterol uptake through lipoproteins (Colardo *et al.*, 2021). qRT-PCR and Western blot analysis showed that neuronal differentiation induced by BET inhibition is paralleled by a transient increase in LDLr, as well as in a sustained expression of LRP1 (Figure 2B,C). A different behavior was observed for 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR), the key and rate-limiting enzyme of cholesterol biosynthetic pathway (Goldstein and Brown, 1990). Indeed, *hmgcr* transcript significantly increases during JQ1-mediated neuronal differentiation. On the contrary, HMGCR protein expression is gradually reduced, especially during terminal differentiation (Figure 2D). Interestingly, an *in vitro* degradation assay revealed that HMGCR degradation rate is enhanced upon JQ1 treatment (Figure 2E). Taken together, these data suggest that *hmgcr* mRNA is increased as a function of nSREBP-2 activation induced by JQ1. Despite this, HMGCR protein expression declines because of intense degradation rate promoted by BET inhibition. Furthermore, CYP46A1 and NPC1 were also

examined. CYP46A1, the enzyme responsible for the turnover of cholesterol in the CNS (*Gamba et al., 2021*), rises in JQ1-treated cells; The amount of NPC1, involved in cholesterol intracellular trafficking (*Li & Pfeffer, 2016*), considerably increases starting from 48 hours of JQ1 treatment (Figure 2F,G). The modulation of CYP46A1 and NPC1 confirms that neuronal differentiation induced by BET blockade is characterized by a lively cholesterol metabolism. Once confirmed that neuronal differentiation induced by BET inhibition was accompanied by rearrangements in cholesterol metabolism, the amount of intracellular cholesterol was also estimated through a colorimetric cholesterol assay. Total, free, and esterified cholesterol levels dramatically increased 72h and 96h after JQ1 stimulation (Figure 2H). These results suggest that cholesterol demand is massive at late stages of neuronal differentiation, supporting the notion that this sterol is essential to assure neurite extension and synaptogenesis promoted by BET inhibition.

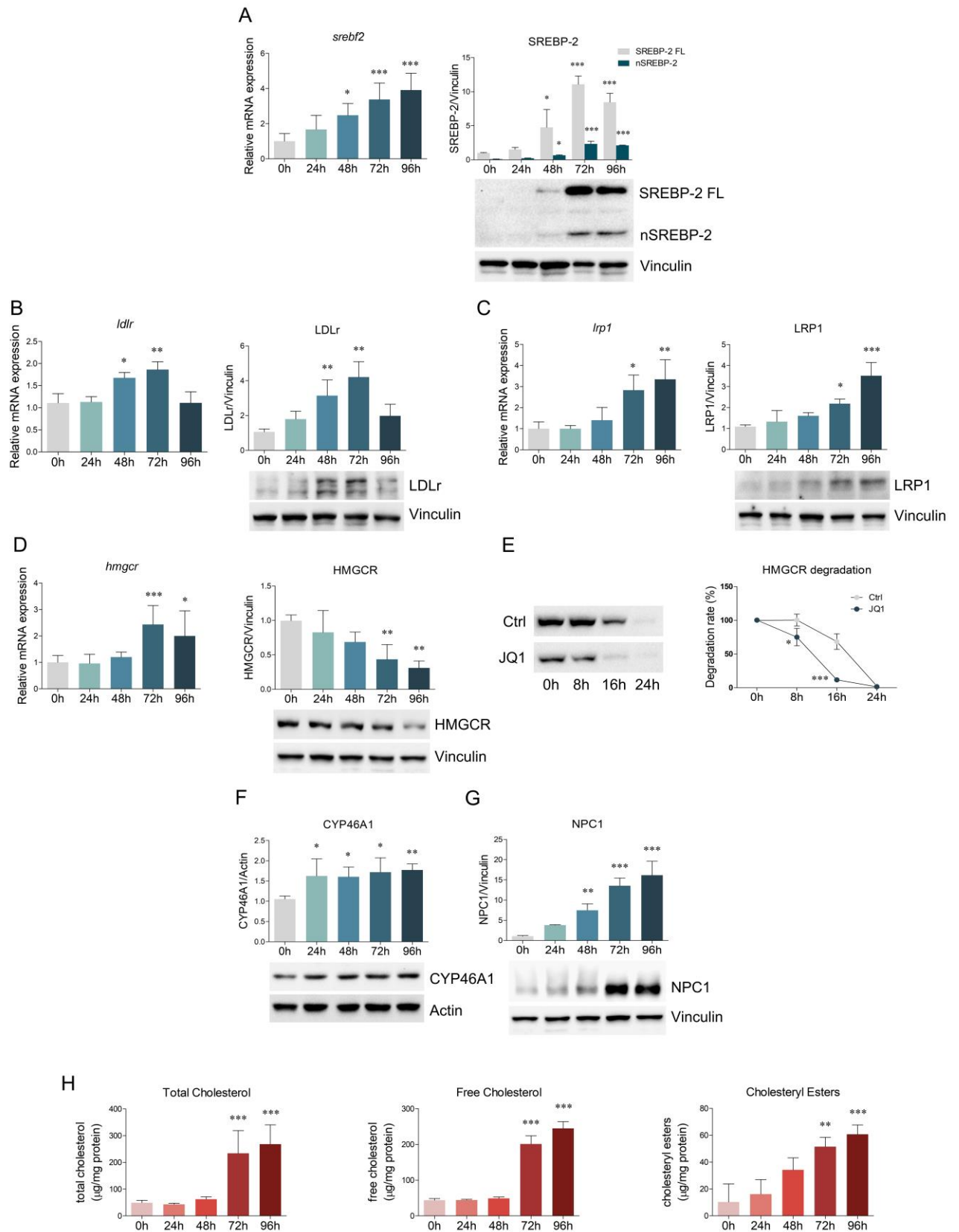


Figure 2. Neuronal differentiation induced by BET blockade leads to a modulation of cholesterol metabolism. (A-D) Time course evaluation of *srebf2*, *ldlr*, *lrp1* and *hmgcr* transcript levels was performed in N1E-115 cells treated with JQ1 (0.1 μ M), up to 96 hours. $n = 6$ independent experiments. (A-D; F-G) Representative Western blot and densitometric analysis of SREBP-2 (FL SREBP-2 (Full-length SREBP-2); nSREBP-2 (nuclear SREBP-2)), LDLr, LRP1, HMGCR, CYP46A1, and NPC1 in N1E-115 cells stimulated with JQ1 (0.1 μ M), in a time course experiment

up to 96 hours. Vinculin and Actin were used as housekeeping proteins to normalize protein loading. $n = 6$ different experiments. **(E)** Representative Western blot and graph showing HMGCR degradation rate of control (Ctrl) and JQ1-treated ($0.1 \mu\text{M}$) N1E-115. $n = 3$ different experiments. **(H)** Time course of intracellular cholesterol levels (total cholesterol, free cholesterol and cholesteryl esters) during neuronal differentiation induced by JQ1. $n = 6$ independent experiments. Data are expressed as means $\pm$ SD. Statistical analysis was performed using one-way ANOVA, followed by Dunnett's post hoc, and two-way ANOVA, followed by Bonferroni's post hoc, where appropriate. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

2.3 Akt and ERK1/2 activation are essential for neuritogenesis and cholesterol modulation promoted by BET blockade

Neuritogenesis is frequently accompanied by the activation of different molecular pathways mediated by Akt and ERK1/2 (Xiao and Liu, 2003; Ryu *et al.*, 2015) kinases. Thus, it was evaluated whether JQ1-induced neuronal differentiation could modulate the activation of these protein kinases. Western blot analysis showed a transient and significant increase in the activating phosphorylation of both Akt and ERK1/2, 24 hours following JQ1 treatment (Figure 3A,B). To evaluate whether these kinases were effectively involved in the differentiation process mediated by BET inhibition, N1E-115 cells were co-treated for 96h with JQ1 and PD98059 ($50 \mu\text{M}$) or Triciribine ($10 \mu\text{M}$), two potent and selective ERK1/2 and Akt inhibitors, respectively. The effects were analyzed through morphological investigations in brightfield and in immunofluorescence by β -III Tubulin visualization. As reported, the pro-differentiating activity of JQ1 is almost totally blocked by the pharmacological inhibition of Akt and ERK1/2, leading to a decrease in the number of neurite-bearing cells and in neurite length (Figure S1; Figure 3C). In addition to β -III Tubulin, the expression levels of GAP-43 and Synaptophysin were also analyzed following co-treatment with PD98059 and Triciribine at 96 hours. Figure 3D highlights that co-administration of these inhibitors strongly hampers the induction of the differentiation markers elicited by JQ1. Importantly, Akt and ERK1/2 inhibition also counteracted cholesterol accumulation promoted by BET inhibition (Figure 3E), suggesting that these kinases crucially influence JQ1-mediated neuronal differentiation by operating a lipid metabolic reprogramming.

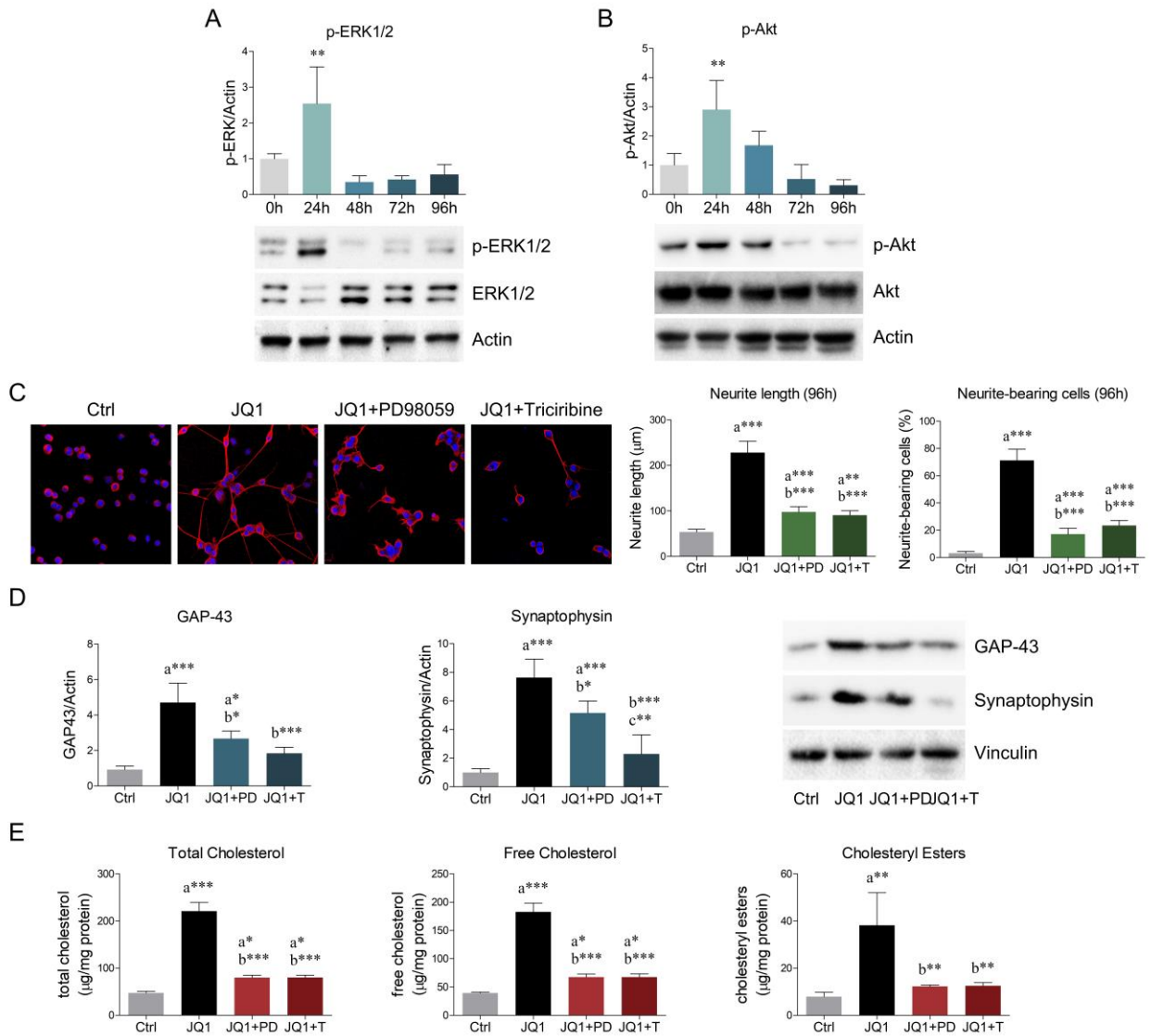


Figure 3. JQ1-mediated neuronal differentiation and cholesterol modulation require Akt and ERK1/2 transient activation in N1E-115 cells. (A-B) Representative Western blot and densitometric analysis of p-ERK1/2 and p-Akt in N1E-115 cells treated with JQ1 (0.1 μM), up to 96 hours. Actin was used as housekeeping protein to normalize protein loading. $n = 6$ independent experiments. (C) Immunofluorescence images of β -III Tubulin (red) in N1E-115 cells treated with vehicle (Ctrl), JQ1 (0.1 μM) or co-treated with JQ1 and PD98059 (50 μM) or with JQ1 and Triciribine (10 μM) for 96 hours. Nuclei were counterstained with DAPI. Graphs indicate the neurite length and the percentage of neurite-bearing cells. $n = 3$ independent experiments. (D) Representative Western blot and densitometric analysis of GAP-43 and Synaptophysin in N1E-115 cells treated as in (C). Vinculin was chosen as loading control. $n = 4$ independent experiments. (E) Graphs show the amount of intracellular cholesterol (total cholesterol, free cholesterol and cholesteryl esters) in N1E-115 treated as in (C). $n = 3$ different experiments. Data are expressed as means $\pm$ SD. Statistical analysis was assessed by using one-way ANOVA, followed by Dunnet's or Tukey's post hoc where appropriate. "a" indicates statistical significance vs Ctrl; "b" indicates statistical significance vs JQ1. "c" indicates statistical significance vs JQ1+PD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

2.4 Neuronal differentiation mediated by BET inhibition is associated to autophagy upregulation

Literature data suggest that BET proteins regulate several metabolic processes, including autophagy (Sakamaki *et al.*, 2017; Li *et al.*, 2019). In particular, BRD4 is a transcriptional repressor of autophagy-lysosomal function (Sakamaki *et al.*, 2017). Since autophagy has been implicated in neuronal differentiation, we analyzed the expression of the main proteins involved in the autophagy-lysosomal process in our model system. Neuronal differentiation induced by BET inhibition is accompanied by a transient increase of Beclin1 protein expression. LC3II/LC3I ratio progressively rises during differentiation (Figure 4A). Unlike the free and cytosolic form (LC3I), LC3II anchors to the autophagosome membrane, thus the conversion rate of LC3I to LC3II represents a valid approach to assess autophagy dynamics and activation (Carlsson *e* Simonsen, 2015). On the other hand, we observed a significant increase in the autophagy substrate SQSTM1/p62 at late stages of JQ1-induced differentiation (Figure 4A). The concomitant increase in LC3II and SQSTM1/p62 could indicate that autophagy induction is related to the enhanced transcription of autophagy substrates. For instance, Sahani *et al.* indicated that SQSTM1/p62 could be induced during autophagy activation (Sahani *et al.*, 2014). Additionally, Li and colleagues demonstrated that SQSTM1/p62 is transcriptionally upregulated by BET inhibition (Li *et al.*, 2019). However, it cannot be excluded that autophagy substrates are accumulated because of autophagy blockade. To clarify this aspect, we performed an autophagy flux experiment by using chloroquine (CQ), a compound capable of inhibiting the fusion of the autophagosome with the lysosome. Figure 4B shows that JQ1 treatment alone induces the increase of LC3-immunodecorated autophagosomes already at 24 hours, as previously suggested by Western blot analysis. Notably, the number and size of autophagosomes was further increased upon CQ co-administration, especially during terminal differentiation (Figure 4B). This suggests that neuronal differentiation induced by BET blockade is associated to the activation of a rapid autophagy flux. Since autophagic activity culminates in an efficient degradation of autophagosome content in the lysosomal compartment, the main proteins of this organelle were analyzed: the lysosomal transmembrane protein LAMP1, and the acid hydrolases, Cathepsin B and Cathepsin D, significantly increase at late stages of JQ1-mediated neuronal differentiation, indicating an efficient lysosomal activity (Figure 4C).

Previous reports highlight that BET proteins are transcriptional repressors of autophagy and lysosomal genes. Coherently, BET blockade enhances autophagy activation and lysosomal function (Sakamaki *et al.*, 2017). Therefore, with the attempt to evaluate whether BET inhibition

could exert transcriptional control on the autophagolysosomal system in our experimental model, we analyzed the mRNA levels of key genes implicated in the autophagy-lysosomal process. As shown in Figure 4D and in Figure S1, *sqstm1*, *lc3b*, *becn1*, *lamp1*, *atg4* and *gabarapl1* significantly increase during differentiation induced by BET inhibition (Figure 4D, Figure S2). Collectively, these results demonstrate that BET inhibition by JQ1 activates autophagy flux and upregulates genes belonging to the autophagy-lysosomal pathway during neuronal differentiation.

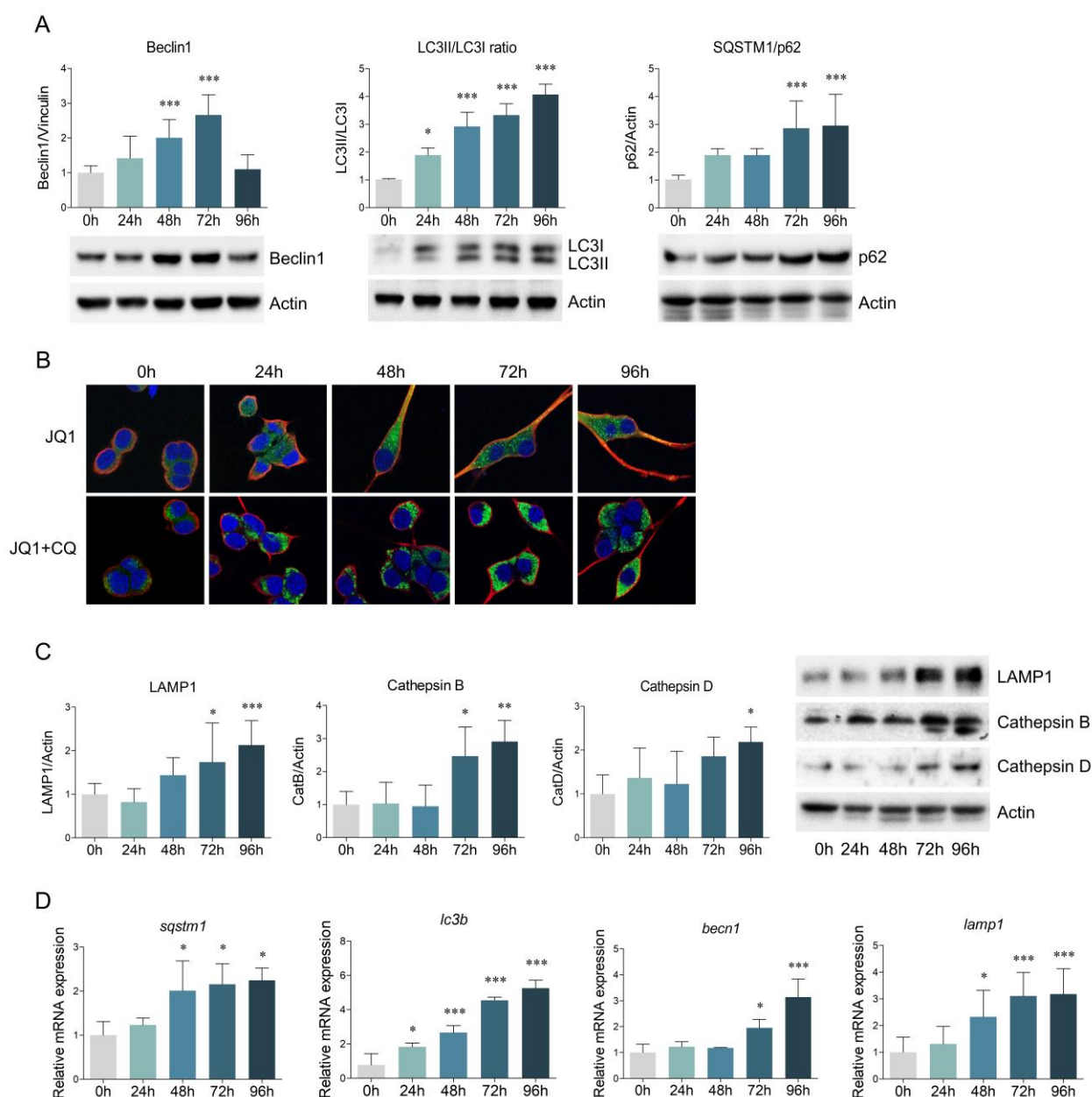


Figure 4. BET inhibition by JQ1 upregulates autophagy during neuronal differentiation in N1E-115 cells. (A) Representative Western blot and densitometric analysis of Beclin1, LC3II and SQSTM1/p62 in N1E-115 treated with JQ1 (0.1 μ M) for 96 hours. Actin was used as loading control. $n = 6$ independent experiments. **(B)** Immunofluorescence images of LC3 (green) and β -III Tubulin (red) in N1E-115 cells treated with JQ1 (0.1 μ M), with or without CQ (10 μ M), up to 96 hours. Nuclei were counterstained with DAPI. **(C)** Representative Western blot and densitometric analysis of LAMP1, Cathepsin B and Cathepsin D in N1E-115 cells treated as in (A). Actin served as a housekeeping protein to normalize protein loading. $n = 8$ different experiments **(D)** qRT-PCR of *sqstm1*, *lc3b*, *becn1*

and *lamp1* transcript levels was performed in N1E-115 cells treated as in (A). $n = 6$ independent experiments. Data are expressed as means $\pm$ SD. Statistical analysis was assessed by using one-way ANOVA, followed by Dunnet's post hoc. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

2.5 Autophagy induction by JQ1 promotes neuronal differentiation and cholesterol mobilization in N1E-115 cells

Autophagy is linked to neuronal differentiation. Accordingly, chemical inhibition of autophagy or knockdown of specific autophagic genes has been shown to suppress neuronal differentiation of N2a cells (Zeng *et al.*, 2008). Therefore, we evaluated whether autophagy was directly involved in the neuronal differentiation induced by BET inhibition in N1E-115 cells. To achieve this objective, we performed a morphological study to visualize neuritogenesis on JQ1-treated N1E-115 cells. Autophagy blockade by CQ attenuates the neurite length as well as the number of neurite-bearing cells induced by JQ1 administration (Figure 5A). This suggests that autophagy is responsible, at least in part, for the pro-differentiating effects of JQ1. Specifically, the marked reduction in the percentage of neurite-bearing cells indicates that JQ1-mediated autophagy induction is particularly relevant in neurite initiation rather than in neurite elongation. Furthermore, the expression of neuronal differentiation markers was also assessed. In contrast to JQ1 treatment, protein expression of GAP-43 and Synaptophysin is considerably reduced when CQ is added to the culture medium (Figure 5B).

We next evaluated whether autophagy activation could be involved in the modulation of cholesterol metabolism induced by BET inhibition. Thus, a colorimetric enzymatic cholesterol assay was performed to assess cholesterol levels at early (24 h) and late (96 h) times of differentiation. No differences were reported for total cholesterol. Conversely, autophagy blockade by CQ treatment significantly reduces free cholesterol, whereas esterified cholesterol is concurrently increased at late stages of neuronal differentiation (Figure 5C). The accumulation of intracellular cholesteryl esters induced by CQ suggest that a significant portion of intracellular free cholesterol may derive from lipophagy during JQ1-mediated neuronal differentiation. Lipophagy results in the degradation of lipid droplets, which are engulfed by autophagosomes and then transported to the lysosome (Yang *et al.*, 2022). During neuronal differentiation, cholesterol biosynthesis is shut down and this lipid is imported from the outside through the activity of LDLr and LRP1 receptors. Then cholesterol-containing lipoproteins enter the cell through receptor-mediated endocytosis mechanisms. Endocytosis and autophagy represent the main pathways for the transport of material to lysosomes (Yang *et al.*, 2022). The uptake of nutrients and

macromolecules then occurs through the formation of vesicles, derived from clathrin-mediated endocytosis, which transport their contents to the early endosomes. Early endosomes, positive for Ras-related protein RAB5A, are converted into late endosomes, expressing RAB7 protein. Here, the cargo can be recycled or delivered to lysosomes for degradation (*Doherty and McMahon, 2009*). This means that autophagy and endocytosis converge into each other. Indeed, autophagosomes fuse with late endosomes to form amphisomes, which are subsequently transported to the lysosome for degradation. In mammalian cells, and particularly in neurons, endocytosis significantly contributes to functional autophagy and the convergence of these two pathways is essential for proper brain function (*Overhoff et al., 2020*). In this context, CQ treatment not only inhibit the fusion of the autophagosome to the lysosome, but also the entire vesicular trafficking (*Lippincott-Schwartz and Fambrough, 1987*). Therefore, absorbed cholesteryl esters could remain trapped inside these vesicles, they are not hydrolyzed by the lysosome and, consequently, they are not made available to the cell in the form of free cholesterol.

In order to specifically identify the vesicular compartment potentially implicated in the accumulation of cholesteryl esters, immunofluorescence analyzes were performed to evaluate the subcellular localization of esterified lipids. Oil Red O staining was employed to label cholesteryl esters. Oil Red O is a fat-soluble dye used for the visualization and measurement of neutral lipids, consisting of triglycerides and cholesteryl esters (*Mehlem et al., 2013*). Co-immunostaining revealed that lipid esters do not localize with both lysosomal (LAMP1) and early endosome (RAB5A) markers (Figure S3). Conversely, CQ treatment determines the appearance of LC3-immunodecorated lipid droplets, meaning that these lipids remain trapped inside the autophagosomes and are not processed by the lysosome (Figure 5D). These results suggest that the induction of autophagy, and particularly lipophagy, is necessary for lipid mobilization required during neuronal differentiation mediated by BET blockade.

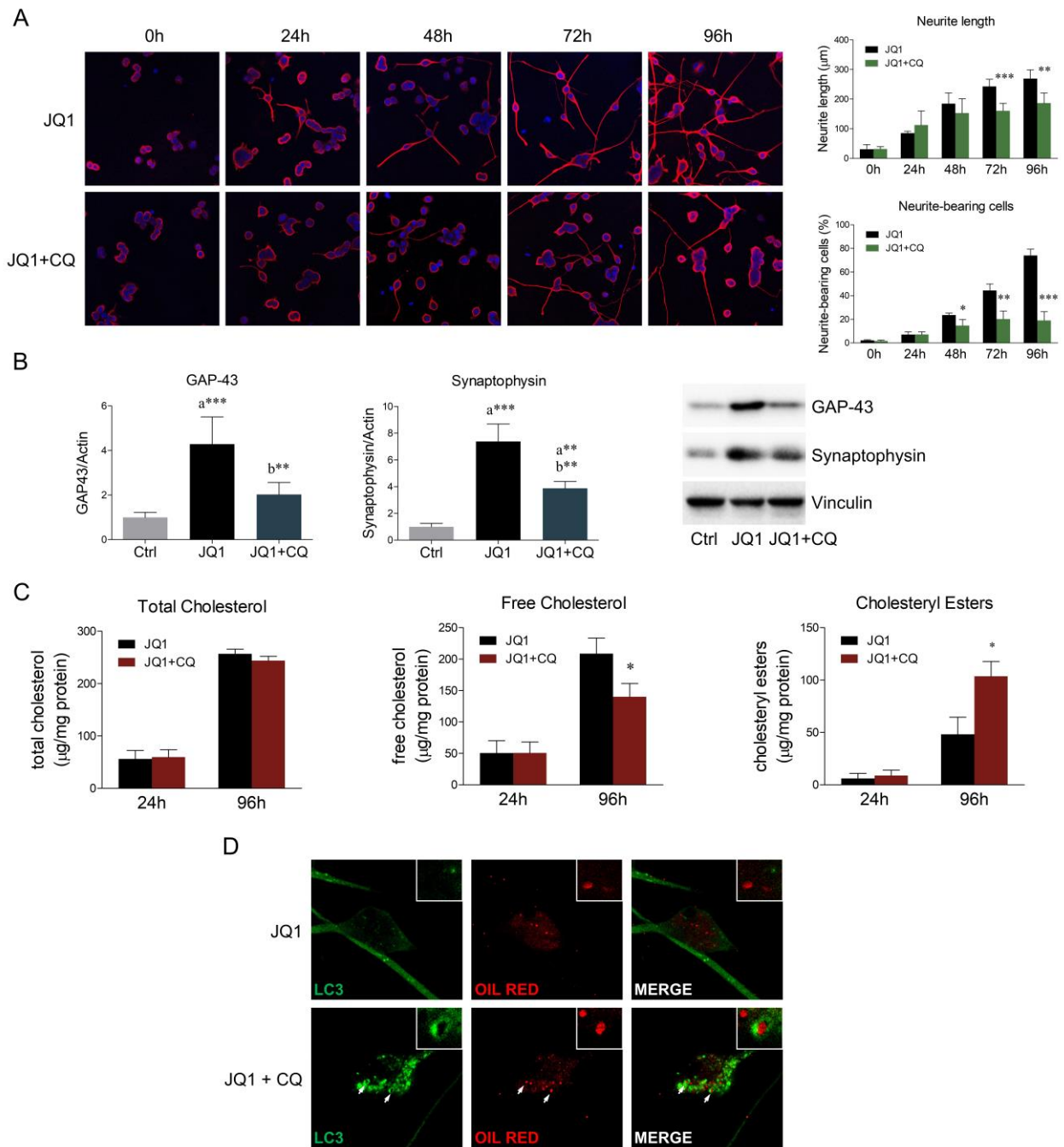


Figure 5. Autophagy modulation by JQ1 influences neuronal differentiation and intracellular cholesterol content. (A) Immunofluorescence of β -III Tubulin (red) in N1E-115 cells treated with JQ1 (0.1 μ M), with or without CQ (10 μ M), up to 96 hours. Nuclei were counterstained with DAPI. Graphs show the neurite length and the percentage of neurite-bearing cells. $n = 5$ independent experiments. (B) Representative Western blot and densitometric analysis of GAP-43 and Synaptophysin in N1E-115 treated with vehicle (Ctrl) or JQ1 (0.1 μ M), with or without CQ (10 μ M), for 96 hours. Vinculin was employed as housekeeping protein to normalize protein loading. $n = 4$ different experiments. (C) Graphs indicate the intracellular cholesterol content (total cholesterol, free cholesterol and cholesteryl esters) in N1E-115 treated as in (A) for 24 and 96 hours. $n = 3$ independent experiments. (D) Co-Immunofluorescence between LC3 (green) and intracellular content of neutral lipids (red) stained with Oil Red O in N1E-115 cells treated as in (A) for 96 hours. $n = 3$ independent experiments. Data are expressed as means $\pm$ SD. Statistical analysis was carried out by using two-way ANOVA, followed by Bonferroni's post hoc, and one-way ANOVA, followed by Tukey's post hoc where appropriate. "a" indicates statistical significance vs Ctrl; "b" indicates statistical significance vs JQ1. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

2.6 BET blockade by JQ1 induces neuronal differentiation and cholesterol accumulation in NSC-34 and ND7/23 cell lines

With the attempt to demonstrate that the effects induced by BET inhibition were not dependent on the specific experimental model selected in this study, the most relevant experiments were recapitulated in NSC-34 and ND7/23 cells, two hybrid cell culture models frequently used in the study of neuritogenesis. To select the most effective dose of JQ1, different concentrations of the drug were tested, and the effects were characterized at terminal differentiation (96 hours for NSC-34, and 72 hours for ND7/23). A dose-dependent effect on neuronal differentiation was observed upon JQ1 treatment in NSC-34 cells, with the most pronounced outcome obtained with the dose of 0.25 μ M (Figure S4). Notably, JQ1 (0.25 μ M) gradually induces neuronal differentiation over time, which is accompanied by a buildup in β -III Tubulin expression (Figure 6A,B).

Similar experiments were performed on ND7/23 cells. Specifically, ND7/23 cells treated with JQ1 for 72 hours show appreciable neurite extension. From a quantitative point of view, the effect is particularly accentuated at the dose of 0.25 μ M (Figure S5A). As for N1E-115 and NSC-34 cells, the extent of neuronal differentiation mediated by BET inhibition in ND7/23 was studied at different experimental times with the selected dose of JQ1, through the quantitative assessment of the neurite-bearing cells and the neurite length (Figure S5B).

JQ1-mediated neuritogenesis is associated to deep rearrangements in cholesterol homeostasis in N1E-115 cells. To assess whether similar effects were preserved in other cell models, a quantitative analysis of cholesterol content was also performed in NSC-34 and ND7/23 cell lines. In both models, neurite extension induced by BET blockade is accompanied by a buildup of total, free and esterified cholesterol during late stages of differentiation (Figure 6C; Figure S5C).

Collectively, these results support the hypothesis that BET inhibition promotes neuronal differentiation in different neuronal cell types, and that this event is paralleled by an increase in the intracellular cholesterol content.

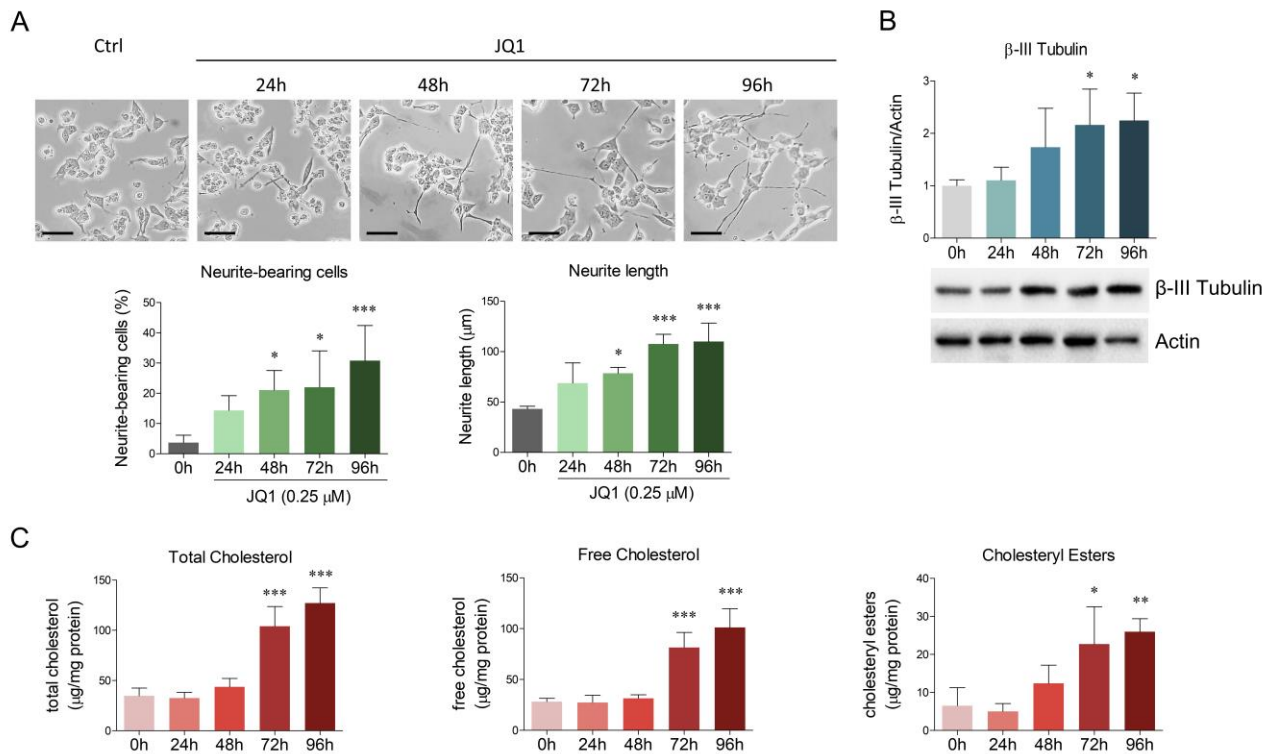


Figure 6. BET inhibition by JQ1 induces neuronal differentiation and modulates intracellular cholesterol content in NSC-34 cells. (A) Brightfield images of NSC-34 cells treated with vehicle (Ctrl) or JQ1 (0.25 μ M) for 96 hours. Graphs represent the percentage of neurite-bearing cells and the neurite length expressed in μ m. $n = 5$ independent experiments. (B) Representative Western blot and densitometric analysis of β -III Tubulin in NSC-34 cells treated as in (A). Actin was used as housekeeping protein to normalize protein loading. $n = 4$ different experiments. (C) Graphs display the amount of intracellular cholesterol content (total cholesterol, free cholesterol and cholesteryl esters) in NSC-34 cells treated as in (A). $n = 3$ independent experiments. Data are expressed as means $\pm$ SD. Statistical analysis was performed by using one-way ANOVA, followed by Tukey's post hoc. "a" indicates statistical significance vs Ctrl; "b" indicates statistical significance vs JQ1. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

2.7 BET inhibition by JQ1 stimulates neuronal differentiation in isolated neural stem cells and promotes adult neurogenesis in mouse brain

In order to reinforce the validity of the results obtained in immortalized cell lines, the pro-differentiating effects mediated by BET inhibition were also tested on isolated neural stem cells (NSC). NSC were isolated from mouse brains and cultured in presence of JQ1 (0.1 μ M) for 48 hours. Subsequently, differentiation toward a neuronal phenotype was estimated through the confocal analysis by visualization of β -III Tubulin-expressing cells. Data showed that BET inhibition increases the number of β -III Tubulin-immunopositive cells (differentiation index) and promotes neuritogenesis, as indicated by the different parameters taken into consideration, such as neurite length, neurite branching and number of neurites (Figure 7A).

Subsequently, the effects of BET inhibition were analyzed on newborn neurons in the adult mouse brain. Specifically, adult neurogenesis was assessed through confocal analysis of Doublecortin (DCX) in the hippocampal DG and in the SVZ of the lateral ventricles, which contain active neurogenic stem cell niches during adulthood. Since DCX is exclusively expressed in developing neurons, it is widely used as a marker of neurogenesis (*Couillard-Despres et al., 2005*). The experiments collected in this study highlight that JQ1 treatment significantly increases the number of DCX-immunopositive cells, both in the DG of the hippocampus and in the SVZ (Figure 7B,C).

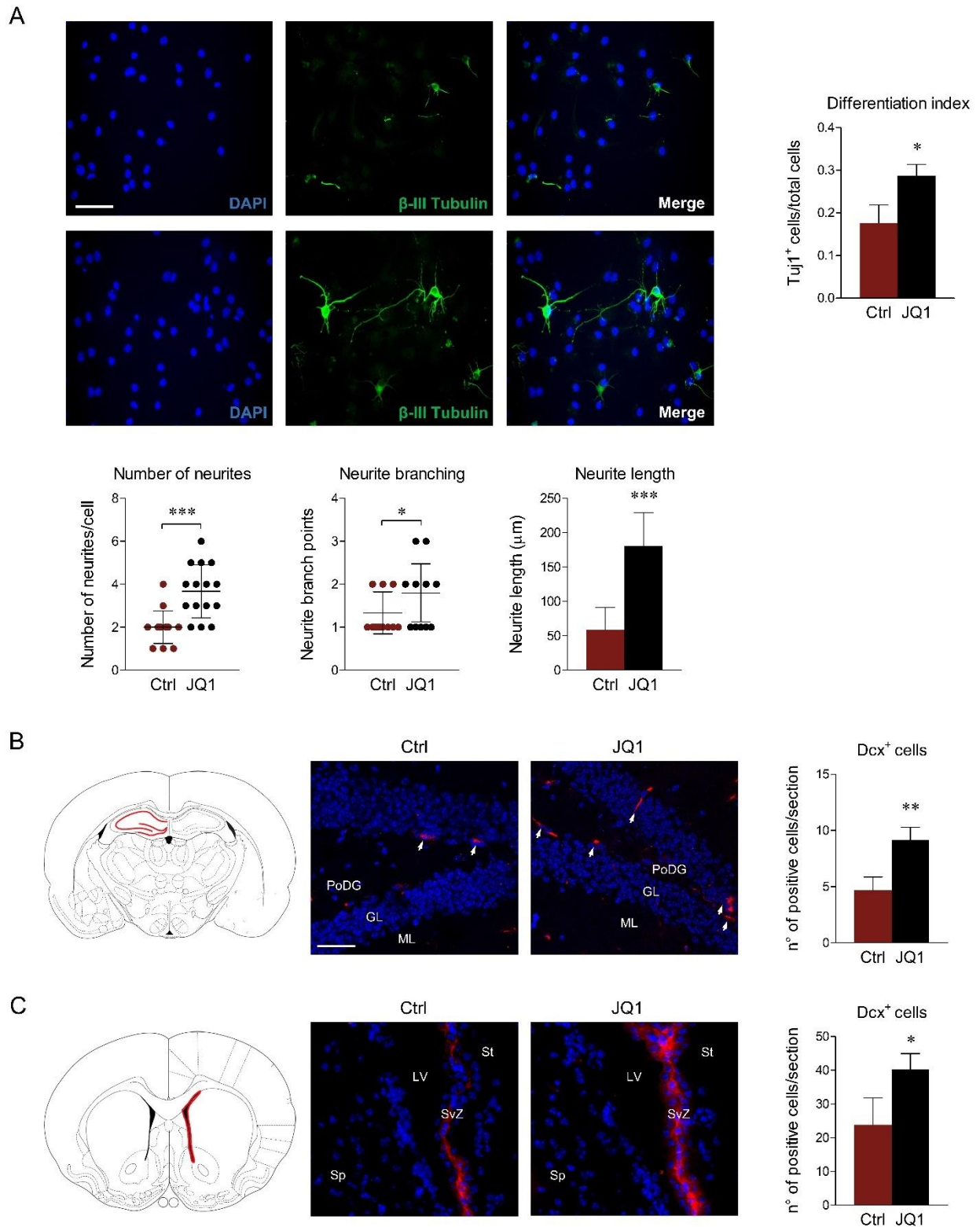


Figure 7. BET inhibition by JQ1 induces neuronal differentiation in a primary NSC line and adult neurogenesis in mouse brain. (A) Immunofluorescence images of β -III Tubulin (green) of NSC treated with vehicle (Ctrl) or JQ1 (0.1 μ M). Nuclei were counterstained with DAPI. Graphs show the ratio of β -III Tubulin-immunopositive cells to the total cell number, the number of neurites per cells, neurite branching and the neurite length in μ m. $n = 3$ independent experiments. **(B)** Bregma from the Mouse Brain Atlas (left panel) showing the SGZ of the DG of the hippocampus (red). Immunofluorescence images (right panel) of DCX (red) in the SGZ of the hippocampal DG. 3-month-old male mice were treated with vehicle (Ctrl) or JQ1 (25 mg/kg) for two weeks. Nuclei were counterstained with DAPI. Graph represents the number of DCX-immunopositive cells, indicated by white arrowheads. $n = 3$ independent experiments.

(C) Bregma from the Mouse Brain Atlas (left panel) showing the SVZ of the lateral ventricles (red). Immunodetection (right panel) of DCX (red) in the SVZ of the lateral ventricles. 3-month-old male mice were treated with vehicle (Ctrl) or JQ1 (25 mg/kg) for two weeks. Nuclei were counterstained with DAPI. Graph shows the number of DCX-immunopositive cells, indicated by white arrowheads. $n = 3$ independent experiments. Data are expressed as means $\pm$ SD. Statistical analysis was evaluated by using unpaired Student's t-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3. Discussion

Neuronal differentiation is a physiological process of neurodevelopment, consisting in the terminal differentiation of NSC, which exhibits specific neuronal features (Zogovic *et al.*, 2015). Interestingly, the process of neurogenesis also occurs in adults, particularly in two specific regions, the SGZ of the DG of the hippocampus and the SVZ of the lateral ventricles, characterized by the presence of neurogenic stem cell niches (Feliciano *et al.*, 2015; Inta *et al.*, 2015). NSC activity is finely regulated by cellular metabolism and by numerous signal transduction cascades (Knobloch and Jessberger, 2017; Hirabayashi *et al.*, 2009). A growing body of evidence indicates that cholesterol metabolism is deeply involved in neuronal differentiation, synaptogenesis and neurotransmission (Suzuki *et al.*, 2007; Lecis *et al.*, 2014; Colardo *et al.*, 2022). In fact, alterations in cholesterol metabolism are associated with several neurodevelopmental and neurodegenerative pathologies (Segatto *et al.*, 2012).

Increasing evidence sustains that neuronal differentiation, as well as cholesterol metabolism, are deeply influenced by the epigenetic control. In this context, the role of BET proteins still remains unclear.

Therefore, in this work, we evaluated the involvement of BET proteins during neuronal differentiation, focusing the attention on the metabolic changes affecting cholesterol metabolism. We observed that inhibition of BET proteins by JQ1 promoted neuritogenesis in all the immortalized cell lines used as experimental models in this study. Indeed, it was possible to appreciate the formation of extensive neurites following JQ1 stimulation which, interestingly, mediates a dose-dependent effect. The greatest outcome was observed at the intermediate concentrations of JQ1 tested in this study (0.1 μ M in N1E-115 cells; 0.25 μ M in ND7/23 and NSC-34 cells). Conversely, neuritogenesis appeared attenuated at higher concentrations. The reason for this dose-response effect could lie in the occurrence of cytotoxic events, phenomena frequently observed when pharmacological compounds are administered at excessive doses. For this reason, after an initial characterization, further analyzes were performed by selecting the most effective doses which, concurrently, resulted free of cytotoxic events.

The pro-differentiating abilities of JQ1 were not only confirmed by morphological analysis, but also evaluating the expression of early and late differentiation markers β -III Tubulin, GAP-43 and Synaptophysin (Sieber-Blum *et al.*, 2006; Rosskoth-Kuhl and Illing, 2014; Calhoun *et al.*, 1996). Our findings strongly support literature data. For instance, it was previously observed that the expression of BET proteins decreases during the differentiation of neural precursors, indicating that a physiological suppression of BET expression is essential for the formation of new neurons. Furthermore, gene expression profiling analysis displayed that BET inhibition induces a specific transcriptional program that enhances neural progenitor cells differentiation into neurons, repressing cell cycle and gliogenesis (Li *et al.*, 2016).

Neuronal differentiation is linked to important changes affecting cholesterol homeostasis.

Consequently, we wondered whether the neuronal differentiation mediated by BET blockade was accompanied by a coherent modulation of cholesterol metabolism. Our data show that proteins and enzymes involved in cholesterol biosynthesis, uptake and intracellular transport are significantly modulated during the differentiation promoted by JQ1. Notably, *hmgcr* mRNA levels increases during JQ1-induced differentiation, whereas its protein levels are progressively reduced because of an enhanced degradation of the enzyme, which compensates for the increased extracellular cholesterol uptake mediated by LDLr and LRP1. These results are in agreement with literature data, supporting the notion that neurons decrease cholesterol synthesis during differentiation and satisfy the need for this sterol by increasing its extracellular intake. In fact, multiple evidence highlights that during development neurons strongly reduce or even abandon cholesterol biosynthesis to preserve the energy required for the generation of action potentials (Pfrieger, 2003). The increased amount of intracellular cholesterol induced by JQ1-mediated neuritogenesis support this hypothesis, suggesting an enhancement of the extracellular cholesterol uptake systems. Noteworthy, BET inhibition by JQ1 has been observed to repress proteins involved in cholesterol metabolism in other cellular contexts. In particular, BET blockade reduces the expression levels of proteins implicated in lipoprotein uptake, such as LDLr (Tonini *et al.*, 2020). This different modulation following JQ1 treatment may depend on the epigenetic landscape that characterizes each cell type.

Considering the strong involvement of Akt and ERK1/2 in the molecular pathways modulating neurite extension, the putative involvement of these two kinases in the effects promoted by BET inhibition was evaluated. The results showed that JQ1 induces a transient hyperactivation of Akt and ERK1/2. However, the inhibition of these kinases suppressed the pro-differentiating effects induced by BET modulation. Interestingly, the reduced neurite extension caused by the pharmacological inhibition of Akt and ERK1/2 is accompanied by a decrease in the intracellular

amount of cholesterol, suggesting that the activities of these kinases are indispensable to guarantee the series of metabolic rearrangements that lead to the modulation of cholesterol network, which are essential in neuronal differentiation. These data further corroborate previous findings, underlining the pivotal role of Akt and ERK1/2 in the induction of early neuritogenesis (*Xiao and Liu, 2003; Ryu et al., 2015*). As an instance, Akt drives signal transduction pathways involved in control of gene transcription and cytoskeletal remodeling, that are required for neurite extension and elongation in different primary cell types, such as hippocampal neurons (*Lim and Walikonis, 2008*), dorsal root ganglion cells (*Tucker et al., 2006; Tucker et al., 2008*) and hypoglossal neurons (*Namikawa et al., 2000*). ERK1/2 phosphorylate different regulators of neuronal differentiation (*Xiao et al., 2003; Ryu et al., 2015*), thus promoting signaling pathways pivotal in all phases of neuronal morphogenesis, such as cell fate specification, neuritogenesis, neurite stabilization, and synapse formation (*Xiao and Liu, 2003*).

Among metabolic processes, autophagy is particularly relevant for neuronal differentiation. Literature data report that autophagy is upregulated during neuronal differentiation of N2a cells and that an impairment of autophagy hinders this physiological process (*Zeng et al., 2008*). Furthermore, autophagy is implicated in embryonic development, as mutations in specific autophagy genes are associated with neural tube defects (*Fimia et al., 2007*).

In our study, JQ1-induced neuronal differentiation correlates with an increased expression of autophagy markers and lysosomal proteins, indicating an induction of autophagy and lysosomal activity.

The increased activity of the autophagic machinery is also associated with the induction of neuronal differentiation. Indeed, autophagy inhibition by CQ led to a marked suppression of neuritogenesis. Importantly, our study reveals that JQ1-mediated autophagy is necessary to mobilize free cholesterol from lipid droplets during neuronal differentiation, indicating that lipophagy strongly contributes to the maintenance of cholesterol homeostasis in neuronal cells.

The pro-differentiating effects mediated by BET inhibition were also confirmed in isolated mouse NSC. Furthermore, JQ1 enhances neurogenesis in the adult mouse brain, both in the hippocampal DG and in the SVZ.

NSC differentiate into mature neurons that are integrated into pre-existing neural networks, thus influencing cognitive processes of learning and memory (*de Miranda et al., 2017*). Alterations in adult neurogenesis result in the loss of existing neurons and in the compromised ability of NSC to self-renew. These alterations characterize several neurodegenerative and neurological diseases, including Parkinson's disease, Alzheimer's disease, Huntington's disease, and schizophrenia (*Horgusluoglu et al., 2017*). Therefore, pharmacological therapies efficiently able to recover

different aspects of adult neurogenesis could be relevant for prospective clinical studies (*Winner and Winkler, 2015*).

Collectively, our data suggest that BET proteins may represent a new level of physiological regulation to control the process of neuronal differentiation. In addition, a deeper understanding of the role played by these epigenetic regulators in neurogenesis could offer significant perspectives for the development of pharmacological approaches aimed at counteracting the loss of neurogenesis that occurs in neurodevelopmental disorders and neurodegenerative diseases.

In this context, BET proteins are promising molecular targets, because of the availability of many inhibitors that modulate their activity: recently, some of these molecules successfully entered clinical trials for other pathologies, demonstrating good efficacy and safety profile (*Shorstova et al., 2021*).

4. Materials and methods

4.1 Cell cultures

Three different immortalized cell lines and a neural stem cell (NSC) line isolated from mouse brain were used in this work: N1E-115 cells, a mouse adrenergic cell line derived from neuroblastoma; NSC-34 cells, a hybrid cell line produced by the fusion of motoneurons derived from the spinal cord of mouse embryos with mouse neuroblastoma cells; ND7/23 cells, derived from the fusion of rat primary dorsal root ganglion neurons with mouse neuroblastoma. Cells were cultured at 5% CO₂ in DMEM medium at high glucose (Merck Life Science, Milan, Italy), containing 10% (v/v) foetal bovine serum (FBS) (Merck Life Science, Milan, Italy), L-glutamine (2 mM), and added with penicillin/streptomycin solution.

In order to evaluate the effects on neuronal differentiation, N1E-115, NSC-34 and ND7/23 cells were initially treated with different doses of JQ1 (Sigma-Aldrich), and the minimum effective dose was selected to carry out the subsequent experiments. N1E-115 cells were treated with JQ1 at concentrations of 0.05 μ M, 0.1 μ M, 0.25 μ M and 0.5 μ M for 96 h. NSC-34 cells were treated with JQ1 at concentrations of 0.1 μ M, 0.25 μ M, 0.5 μ M and 1 μ M and induced to differentiate for 96 h. ND7/23 cells were treated with JQ1 at concentrations of 0.1 μ M, 0.25 μ M, 0.5 μ M and 1 μ M in DMEM medium with 0.5% FBS and induced to differentiate for 72 h. NSC cells were treated with JQ1 at concentration of 0.1 μ M for 48 h.

To evaluate the involvement of specific protein kinases in the effects induced by JQ1, N1E-115 cells were treated with the compound PD98059 (Tocris) at the concentration of 50 μ M, and with Triciribine (Calbiochem) at the concentration of 10 μ M, up to 96 h.

To assess the role of autophagy in neuronal differentiation induced by BET inhibition, N1E-115 cells were co-treated with CQ at the concentration of 10 μ M for 2 h to inhibit the late stage of autophagic process.

4.2 Animals study experimental design

Three months old-mice (Charles River, Italy) were housed in groups of five and maintained under controlled temperature (20 ± 1 °C), humidity ($55 \pm 10\%$), and illumination (12/12 h light cycle with lights on at 07:30 am). Food and water were provided *ad libitum*. All mice were held in quarantine for 2 weeks before the experiments. Tubes for tunneling and nesting materials (paper towels) were routinely placed in cages as environmental enrichment. All procedures involving animal care or treatments were approved by the Italian Ministry of Health and performed in compliance with the guidelines of the Italian Ministry of Health (according to the Legislative Decree 116/92), the Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Animals were then randomized and divided into two groups: control (mice treated with vehicle) and JQ1 (25 mg/kg body weight of JQ1, daily administered by intraperitoneal injections during the whole experimental period). After two weeks, mice were sacrificed, and their brains quickly collected. Brains were frozen in cooled isopentane with liquid nitrogen and stored at -80° for subsequent morphological analysis.

4.3 Lysate preparation and Western blot analysis

N1E-115 and NSC-34 cells were sonicated (duty cycle 20%, output 3) in sample buffer (0.125 M Tris-HCl containing 10% SDS, protease inhibitor cocktail, pH 6.8) for 30 s in order to obtain a total lysate. The samples were then centrifuged at 12000 g for 7 min to remove cellular debris. Lowry's methods were used to evaluate protein concentration. Then, Laemmli buffer was added, and the samples were boiled at 95°C for 3 minutes. Protein extracts (20 μ g of proteins) were resolved on SDS-PAGE, and the transfer onto nitrocellulose membrane was performed using a

turbo trans-blot transfer system (Biorad Laboratories, Milan, Italy). Subsequently, the membrane was incubated at room temperature for 1 h with 5% fat-free milk powder in Tris-buffered saline (25 mM Tris-HCl, 138 mM NaCl, 27 mM KCl, 0.05% Tween-20, pH 6.8) and probed overnight at 4°C with the following primary antibodies: anti- β -III Tubulin (Santa Cruz Biotechnology, Dallas, TX, USA, sc-80005, dilution 1:10000); anti-GAP-43 (Santa Cruz Biotechnology, Dallas, TX, USA, sc-17790, dilution 1:1000); anti-Synaptophysin (Santa Cruz Biotechnology, Dallas, TX, USA, sc-17750, dilution 1:2000); anti-SREBP-2 (Abcam, Cambridge, UK, ab28482, dilution 1:1000); anti-LDLr (Abcam, Cambridge, UK, ab30532, dilution 1:1000); anti-LRP1 (Santa Cruz Biotechnology, Dallas, TX, USA, sc-25469, dilution 1:1000); anti-HMGCR (Abcam, Cambridge, UK, ab174830, dilution 1:1000); anti-CYP46A1 (Santa Cruz Biotechnology, Dallas, TX, USA, sc-136148, dilution 1:1000); anti-NPC1 (Novus Biologicals, Centennial, CO, USA, NB400-148, dilution 1:2000); anti-P-ERK1/2 (Santa Cruz Biotechnology, Dallas, TX, USA, sc-7383, dilution 1:1000); anti-tot-ERK1/2 (Santa Cruz Biotechnology, Dallas, TX, USA, sc-94, dilution 1:3000); anti-P-Akt (Santa Cruz Biotechnology, Dallas, TX, USA, sc-7985, dilution 1:1000); anti-tot-Akt (Santa Cruz Biotechnology, Dallas, TX, USA, sc-8312, dilution 1:1000); anti-Beclin1 (Santa Cruz Biotechnology, Dallas, TX, USA, sc-11427, dilution 1:1000); anti-LC3 (Sigma-Aldrich, Milan, Italy, L7543, dilution 1:2000); anti-SQSTM1/p62 (Santa Cruz Biotechnology, Dallas, TX, USA, sc-48402, dilution 1:1000); anti-Lamp1 (Abcam, Cambridge, UK, ab24170, dilution 1:1000); anti-Cathepsin B (Santa Cruz Biotechnology, Dallas, TX, USA, sc-365558, dilution 1:1000); anti-Cathepsin D (Santa Cruz Biotechnology, Dallas, TX, USA, sc-10725, dilution 1:1000); anti-Actin (Santa Cruz Biotechnology, Dallas, TX, USA, sc-47778, dilution 1:10000); anti-Vinculin (Sigma-Aldrich, Milan, Italy, V9264, dilution 1:10000). Subsequently, the membranes were probed for 1 h with HRP-conjugated secondary anti-mouse or anti-rabbit antibodies (Bio-Rad Laboratories, Milan, Italy). Protein-bound antibodies were visualized using clarity ECL Western blotting (Bio-Rad Laboratories, Milan, Italy, #1705061), and chemiluminescence was recorded using ChemiDoc MP system (Bio-Rad Laboratories, Milan, Italy). The obtained images were subjected to densitometric analysis using ImageJ software (National Institutes of Health, Bethesda, MD, USA) for Windows. All samples were normalized for protein loading with Actin or Vinculin, used as housekeeping proteins. Densitometric calculations derived from the ratio between the protein band intensity and the respective housekeeping protein.

4.4 RNA extraction and Real-Time PCR

mRNA analysis was performed as previously reported (Pallottini et al., 2020; Segatto et al., 2020). Briefly, total RNA from N1E-115 cells was extracted with TRI Reagent (Merck Life Science, Milan, Italy) according to the manufacturer's instructions. DNase treatment (Ambion, Thermo Fisher Scientific, Milan, Italy) was performed, and then RNA was purified using an RNA clean up Kit (Zymo, Italy). RNA was successively reverse transcribed to cDNA through a High-Capacity cDNA Reverse Transcription Cell Kit (Applied Bio-System, Foster City, CA, USA) and used for qPCR analysis. Primers used in qRT-PCR were: *srebf2* forward 5'-GGCCTCTCCTTTAACCCCTT-3' and reverse 5'-ACCATTTACCAGCCACAGGA-3'; *ldlr* forward 5'-ACATCTGCAAGGACCTCAAGA-3' and reverse 5'-CTCACACTTGTAGCTGCCTTC-3'; *lrp1* forward 5'-TTGCGCTGACCCTATTTGAAG-3' and reverse 5'-GTTGACTTTGCAGGGGTGATT-3'; *hmgcr* forward 5'-CCTTGTTACGCTCATAGTCG-3' and reverse 5'-CTTGCTCAATGTCCATGCTGA-3'; *sqstm1* forward 5'-GCTCCACCAGAAGATCCCAA-3' and reverse 5'-TTTCTGGGGTAGTGGGTGTC-3'; *lc3b* forward 5'-GTCCGAGAAGACCTTCAAGC-3' and reverse 5'-AAGCGCCGTCTGATTATCTT-3'; *becn1* forward 5'-AGGATGGTCTCTCTCGAAGAT-3' and reverse 5'-GATCAGAGTGAAGCTATTAGCACTTTC-3'; *lamp1* forward 5'-TCTAGTGGGAGTTGCGGTATC-3' and reverse 5'-CACTAGGGCATCAGGAAGAGT-3'; *atg4* forward 5'-AAGGAAGTTTTCCCGATTG-3' and reverse 5'-CCACAGCGCAGCATACAT-3'; *gabara β 1* forward 5'-CATCGTGGAGAAGGCTCCTA-3' and reverse 5'-TCCTCAGGTCTCAGGTGGAT-3'; *gapdh* (chosen as reference gene) forward 5'-AACATCAAATGGGGTGAGGCC-3' and reverse 5'-GTTGTCATGGATGACCTTGGC-3'. Each biological sample was run in triplicate with SYBR green IQ reagent (Bio-Rad Laboratories, Milan, Italy) and using the CFX Connect detection system (Bio-Rad Laboratories, Milan, Italy).

4.5 Immunofluorescence and Oil Red O staining

Immunofluorescence of N1E-115, NSC-34, ND7/23 and NSC cells was performed as previously reported (Tonini et al., 2020). Cells were seeded on poly-L-lysine (Sigma-Aldrich, P6282-5MG)-coated coverslips in 6-well plates, then fixed in paraformaldehyde (4% in PBS) and probed

overnight with primary antibodies: anti- β -III Tubulin (Santa Cruz Biotechnology, Dallas, TX, USA, sc-80005, dilution 1:200); anti-LC3 (Sigma-Aldrich, Milan, Italy, L7543, dilution 1:400); anti-Lamp1 (Abcam, Cambridge, UK, ab24170, dilution 1:100); anti-RAB5A (Santa Cruz Biotechnology, Dallas, TX, USA, sc-598). Next, cells were incubated for 1 h with goat anti-mouse secondary antibody Alexa Fluor 555 or Alexa Fluor 488 (ThermoFisher Scientific, Waltham, MA, USA, A28180 and A27034, respectively) and with goat anti-rabbit secondary antibody Alexa Fluor 488. DAPI staining was performed to visualize nuclei, and coverslips were finally mounted with Fluoroshield mounting medium (Sigma-Aldrich, F6182). The preparations were examined under confocal microscopy (TCS SP8; Leica, Wetzlar, Germany) and the images were acquired at 40X or 63X magnification using Leica LAS X Software (Leica Microsystems, Buccinasco, Italy). To evaluate the localization of neutral lipid following CQ blockade in N1E-115 cells, co-immunofluorescence was performed between the different organelle markers taken into consideration (LC3, LAMP1 and RAB5A) and the neutral lipids, stained by Oil Red O. Oil Red O staining was performed as previously reported (*Tonini et al., 2020*). Once immunofluorescence has been performed as described above and before slides mounting, cells were incubated with 60% isopropanol for 5 min and then washed with distilled water. Subsequently, cells were probed with 1 mL of Oil Red O working solution (Sigma-Aldrich, O1391-250ML) for 15 min at room temperature by putting the 6-well plate onto an orbital rotator shaker. After the incubation with the staining solution, wells were washed three times with distilled water to eliminate the excess stain. Coverslips were finally mounted with Fluoroshield mounting medium, and Oil Red O autofluorescence was visualized via confocal microscopy. The images were captured using Leica LAS X Software with a 63X magnification.

In vivo morphological evaluation was performed on OCT frozen brains. Transverse, 10- μ m-thick sections were cut by a cryostat and collected on Superfrost Plus slides (BioOptica).

For immunofluorescence analysis of brains, 10- μ m frozen sections were blocked with 10% Normal Goat Serum (NGS, Vector Laboratories) in PBS with 2.5% Triton-X 100, and then incubated overnight at 4°C with anti-DCX (Santa Cruz Biotechnology, Dallas, TX, USA, sc-271390, dilution 1:100). Sections were thoroughly rinsed with PBS, then incubated for 1 h at room temperature with PBS containing 5% NGS and goat anti-mouse secondary antibody Alexa Fluor 555. Nuclei were counterstained with DAPI and then slides were mounted with Fluoroshield mounting medium. Brain slices were observed under confocal microscopy and images were captured at 40X magnification.

4.6 Cholesterol quantification

Cholesterol quantification was performed by using a colorimetric assay (Cholesterol Quantitation Kit, MAK043, Sigma-Aldrich, Milan, Italy) following the manufacturer's instructions.

4.7 Degradation assay

HMGR degradation assay was carried out as in *Segatto et al., 2014*. Briefly, N1E-115 cells were treated with vehicle (Ctrl) or JQ1 (0.1 μ M) at 96 h, as already reported. Then, cells were lysed in ice cold 0.01 M Tris-HCl (pH 7.4), 0.150 M sucrose and incubated at 37°C. Total protein concentration was assessed through the Lowry's method. 50 μ g homogenate was employed for each reaction. After each chosen time interval, the incubation was stopped and the reaction was blocked by adding an equal volume of sample buffer (as above mentioned). Then, all the samples were boiled for 3 minutes and used for Western blot analysis.

4.8 Quantitative evaluation of neuronal morphology

Neuronal morphology in N1E-115, NSC-34, ND7/23 and NSC cells was estimated by capturing images with an inverted and confocal microscope. Morphometric analysis was performed by using ImageJ software for Windows. The differentiation parameters considered are represented by neurite-bearing cells (expressed as percentage calculated by dividing the number of differentiated cells with the total number of cells per field), neurite length (expressed in μ m), differentiation index (expressed as the ratio between the number of β -III Tubulin immunopositive cells and the number of total cells), number of neurites per cell, and neurite branching. Cells were considered differentiated when they had at least one neurite with length equal to or greater than the diameter of the soma. Morphological assessment was carried out on at least five different experiments, in which at least three images were analyzed.

4.9 Statistical analysis

All the results presented in this study were expressed as mean $\pm$ SD (standard deviation). One-way analysis of variance (ANOVA) followed by Tukey's or Dunnet's post hoc test was used. When presenting two variables, two-way ANOVA followed by Bonferroni post hoc test was employed. Values of $p < 0.05$ were considered to be statistically different. Statistical analysis was performed using GraphPad Prism 5 (GraphPad, La Jolla, CA, USA) for Windows.

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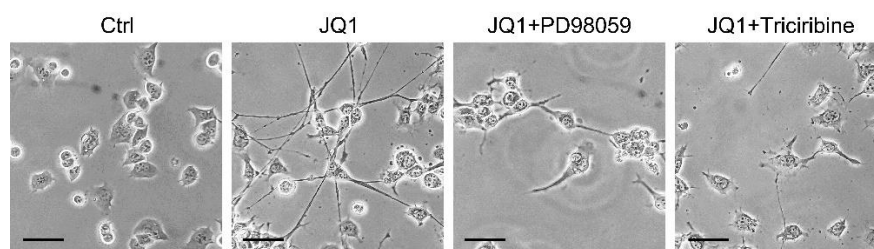
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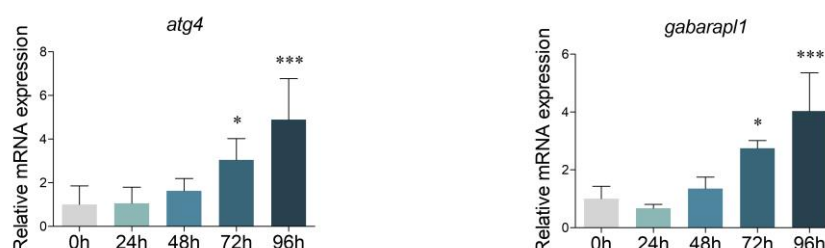
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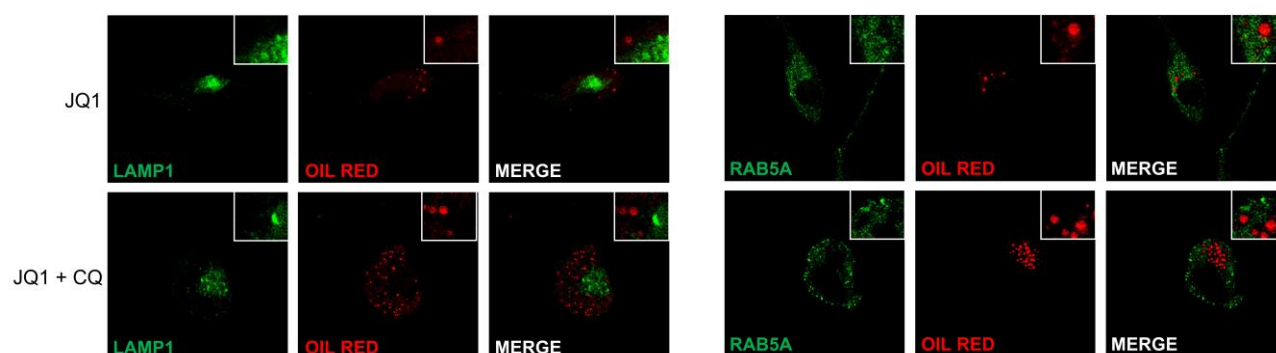
Supplementary figures



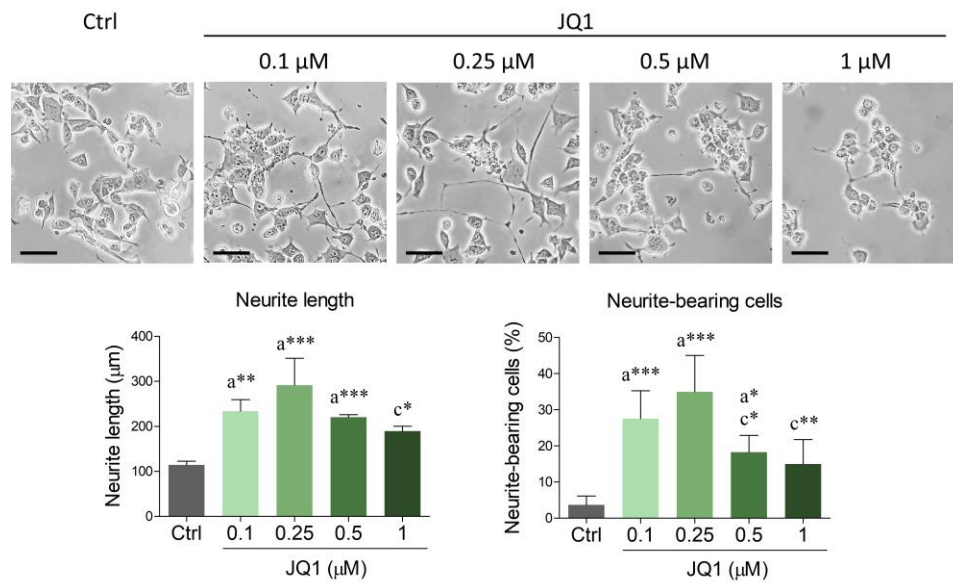
Supplementary figure 1. BET inhibition-mediated neuronal differentiation requires Akt and ERK1/2 transient activation in N1E-115 cells. Brightfield images of N1E-115 cells treated with vehicle (Ctrl) or JQ1 (0,1 μ M) or co-treated with JQ1 and PD98059 (50 μ M) or with JQ1 and Triciribine (10 μ M) at 96 hours.



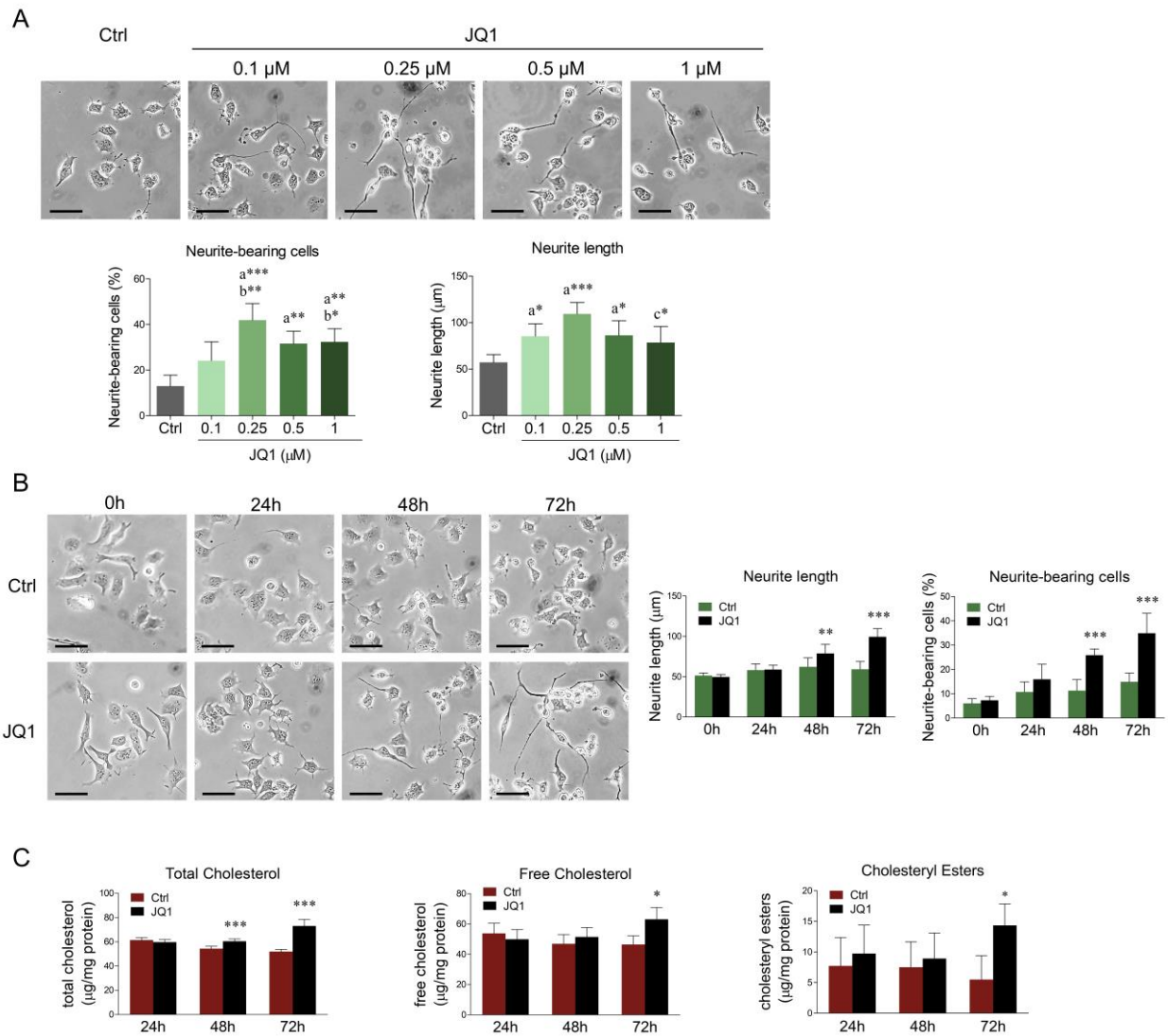
Supplementary figure 2. JQ1 upregulates the transcription of genes involved in autophagy. qRT-PCR of *atg4* and *gabarapl1* transcript levels was performed in N1E-115 control cells (0 h) or JQ1 (0.1 μ M), up to 96 hours. $n = 6$ independent experiments. Data are expressed as means $\pm$ SD. Statistical analysis was assessed by using one-way ANOVA, followed by Dunnet's post hoc. * $p < 0.05$; *** $p < 0.001$.



Supplementary figure 3. Neutral lipids do not become trapped in lysosomes or early endosomes following vesicular trafficking blockage with CQ. Immunofluorescence images of LAMP1, used as a lysosome marker, (green; left panel) or RAB5A, used as a marker of early endosomes, (green; right panel) and intracellular content of neutral lipids (red) stained with Oil Red O in N1E-115 cells treated with JQ1 (0.1 μ M), with or without CQ (10 μ M) at 96 hours. $n = 3$ independent experiments



Supplementary figure 4. JQ1-mediated BET inhibition promotes neuronal differentiation in NSC-34 cells. Brightfield images of NSC-34 cells treated with vehicle (Ctrl) or JQ1 (0.1 μM, 0.25 μM, 0.5 μM, 1 μM) for 96 hours. Graphs represent the neurite length expressed in μm and the percentage of neurite-bearing cells. $n = 3$ independent experiments. Data are expressed as means $\pm$ SD. Statistical analysis was performed by using one-way ANOVA, followed by Tukey's post hoc. "a" indicates statistical significance vs Ctrl; "c" indicates statistical significance vs JQ1 0.25 μM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.



Supplementary figure 5. BET inhibition by JQ1 induces neuronal differentiation and increases cholesterol content in ND7/23 cells. (A) Brightfield images of ND7/23 cells treated with vehicle (Ctrl) or JQ1 (0.1 μ M, 0.25 μ M, 0.5 μ M, 1 μ M) for 72 hours. Graphs represent the percentage of neurite-bearing cells and the neurite length. $n = 5$ independent experiments. (B) Brightfield images of ND7/23 cells treated with vehicle (Ctrl) or JQ1 (0.25 μ M) for 72 hours. Graphs represent the neurite length expressed in μ m and the percentage of neurite-bearing cells. $n = 4$ different experiments. (C) Intracellular cholesterol content (total cholesterol, free cholesterol and cholesteryl esters) in ND7/23 cells treated as in (B). $n = 3$ independent experiments. Data are expressed as means $\pm$ SD. Statistical analysis was assessed by using one-way ANOVA, followed by Tukey's post hoc, and two-way ANOVA, followed by Bonferroni's post hoc where appropriate. "a" indicates statistical significance vs Ctrl; "b" indicates statistical significance vs JQ1 0.1 μ M; "c" indicates statistical significance vs JQ1 0.25 μ M. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.



Review

Neurotrophins as Key Regulators of Cell Metabolism: Implications for Cholesterol Homeostasis

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Abstract: Neurotrophins constitute a family of growth factors initially characterized as predominant mediators of nervous system development, neuronal survival, regeneration and plasticity. Their biological activity is promoted by the binding of two different types of receptors, leading to the generation of multiple and variegated signaling cascades in the target cells. Increasing evidence indicates that neurotrophins are also emerging as crucial regulators of metabolic processes in both neuronal and non-neuronal cells. In this context, it has been reported that neurotrophins affect redox balance, autophagy, glucose homeostasis and energy expenditure. Additionally, the trophic support provided by these secreted factors may involve the regulation of cholesterol metabolism. In this review, we examine the neurotrophins' signaling pathways and their effects on metabolism by critically discussing the most up-to-date information. In particular, we gather experimental evidence demonstrating the impact of these growth factors on cholesterol metabolism.

Keywords: BDNF; cholesterol; metabolism; neurotrophins; NGF; p75NTR; TrkA; TrkB

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1. Introduction

At the end of the 19th century, the knowledge concerning neuroscience research underwent deep changes starting from the hypothesis that nerve cells need trophic support [1]. By a rare combination of intuition and scientific interpretation, this assumption was demonstrated by Rita Levi-Montalcini, Viktor Hamburger and Stanley Cohen in the early 1950's with the discovery of nerve growth factor (NGF), the first growth factor characterized [2,3]. This was embodied in a conceptual postulate, known as neurotrophic theory, which sustains a crucial role of NGF and other neurotrophic factors in the regulation of neuronal survival, differentiation, and function [1].

Even though they were initially regarded as signaling molecules with specific tropism for neuronal cells, compelling evidence indicates that NGF and the other related factors, called neurotrophins (NTs), can promote the survival and the growth of several non-neuronal cells. Importantly, NTs affect a number of metabolic processes associated to the control of glucose levels, autophagy, oxidative stress, energy homeostasis and lipid metabolism [1,4]. During the last few years, experimental findings highlighted a role for NTs in cholesterol metabolism regulation. Cholesterol plays crucial roles in cell physiology, as it assures the proper functioning of cell membranes and regulates different intracellular molecular pathways involved in multiple biological processes [5]. Thus, it is

not surprising to discover that signaling factors such as NTs, implicated in the growth, survival and differentiation of both neuronal and non-neuronal cells, could influence cell homeostasis by modulating, at least in part, cholesterol metabolism.

The purpose of this review article is to collect and critically discuss the current knowledge about the role exerted by NTs in the regulation of cell metabolism, with particular emphasis on cholesterol metabolism. In addition, the most relevant pathways involved in NTs signaling and cholesterol homeostasis are also described, and integrated with the most up-to-date information.

2. Neurotrophins: From Cell Signaling to Metabolic Processes

NTs are a small family of growth factors significantly involved in the regulation, development and function of the nervous system [6,7]. In 1951, Nobel laureate Rita Levi-Montalcini discovered the nerve growth factor (NGF), a neurotrophin able to enhance the growth of sensory and sympathetic neurons [8,9]. Later, other important NTs were discovered, as the brain-derived neurotrophic factor (BDNF), the neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) [10–13]. NTs are initially synthesized as pro-neurotrophins (pro-NTs), high molecular weight precursors of 240–260 amino acids. Pro-NTs are proteolytically cleaved by proteases to obtain mature neurotrophins with a molecular weight of approximately 13–14 kDa [14,15]. The production of mature NTs is cell-tissue dependent and their expression pattern may vary according to cell context and physiopathological condition [7]. For instance, proprotein convertase subtilisin/kexin 3 (PCSK9, or furin) is expressed in most mammalian cells, while proprotein convertase subtilisin/kexin 1 (PCSK1, or NEC1) and proprotein convertase subtilisin/kexin 2 (PCSK2, or NEC2) preferentially show neuronal expression. These enzymes expression is subjected to elegant physiological regulations, and a considerable amount of pro-NTs can be directly secreted in the extracellular milieu in specific contexts. Once secreted, mature NTs influence a variety of aspects related to the development and the functioning of the nervous system, such as neurite extension, axonal growth and neuronal differentiation, myelination, synaptic plasticity, survival and apoptosis [6,7,16]. In the extracellular space, pro-NTs may undergo proteolytic processing by the activity of plasmin and/or matrix metalloproteases (for extensive review, please see ref. [17–19]). Interestingly, it is now well-established that, independently from their mature forms, pro-NTs exert biological activity and may display different, even opposite, effects if compared to their mature counterparts [6,7].

2.1. Neurotrophin Receptors and Signal Transduction

The cellular effects mediated by NTs and their precursors are strictly dependent on the binding of two different classes of membrane receptors: tropomyosin-receptor kinases (Trks) and the pan-neurotrophin receptor p75 (p75NTR) (Figure 1). Trks are transmembrane glycoproteins with an extracellular neurotrophin-binding domain comprising two cysteine-rich regions separated by a leucine-rich repeat, two tandem immunoglobulin G (IgG)-like domains located nearby the plasma membrane, a single-pass transmembrane region, and an intracellular domain with intrinsic tyrosine kinase activity. NTs bind the second IgG-like domain, resulting in Trk receptor homodimer formation, and subsequent triggering of tyrosine kinase activity by autophosphorylation [16]. Each neurotrophin selectively binds a different Trk: NGF activates TrkA; BDNF and NT-4 bind to TrkB; whereas NT-3 preferentially elicits TrkC activation [16,20]. However, Trks signaling is made more intricate by heterologous binding. For instance, it has been demonstrated that NT-3 and NT-4 can also bind to and activate TrkA, as well as NT-3, may induce the activation of TrkB [13,20]. The complexity of NTs signaling is also increased by the expression of Trks splicing variants, which have different properties if compared to the full-length (FL) receptors. In this context, several reports demonstrated the existence of Trk receptors with deletions in both the extracellular and intracellular domains [21–23]. These specific isoforms may act as dominant negative modulators, NTs

sequestrants or trigger alternative signaling pathways when compared to FL receptors [24]. Experimental evidence highlighted that an 18 bp deletion in the extracellular domain alters TrkA activation by NT-3. In contrast, exon 9 deletion renders TrkB less prone to NT-4 and NT-3 stimulation [22,23]. TrkB.T1 is the most commonly expressed truncated isoform of TrkB [25,26] and it shows the same binding affinity to BDNF if compared to TrkB FL. However, its intracellular domain lacks the tyrosine kinase domain, resulting in dimerization inability and consequently, in the classic TrkB FL signal transduction pathways activation. TrkB.T1 is particularly expressed in the adult nervous system [25,27], predominantly in astrocytes [28–30]. The exact biological effects of TrkB.T1 signaling are not fully elucidated [31]. However, it has been observed that it may regulate intracellular calcium entry, as well as small Rho GTPase activity in astrocytes [28,32,33]. In their FL forms, the Trks act mainly through three major transduction pathways: phospholipase C- γ 1 (PLC- γ 1), Ras and phosphatidylinositol 3-kinase (PI3K) [34]. Trk phosphorylation at Tyr785 residue generally leads to the activation of the PLC- γ 1 signaling pathway [24]. Indeed, PLC- γ 1 catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to yield diacylglycerol (DAG) and inositol trisphosphate (IP3). DAG activates protein kinase C (PKC), whereas IP3 binds to its receptors and leads to the release of Ca^{2+} from the endoplasmic reticulum (ER) stores which, in turn, activates the Ca^{2+} -calmodulin-dependent protein kinases. Several findings demonstrated that the involvement of the PLC- γ 1 pathway in Trks activation is crucial for the induction and maintenance of synaptic plasticity [35]. In addition, PLC- γ 1 influences key cellular processes, such as proliferation and differentiation, in both neuronal and non-neuronal cells [6,36–40]. The activation of Ras pathway requires the NT binding to Trk receptors, their phosphorylation at Tyr490 residue and the subsequent recruitment/phosphorylation of the adaptor protein Shc. Phosphorylated Shc binds to the Grb2-SOS complex, activating the guanine nucleotide exchange factor SOS. SOS removes GDP from the small GTPase Ras, which is now able to bind to GTP and switch to the active form. Subsequently, Raf kinase is activated by Ras, and in turn, phosphorylates ERK. ERK regulates the activation of several downstream substrates and mediates different cellular effects such as proliferation, differentiation, survival, cell growth and apoptosis [6,41]. Notably, ERK can enter the nucleus to regulate the activation of several transcription factors, including cAMP response element-binding protein (CREB), which controls transcriptional changes required for neuronal differentiation, neurite outgrowth and synaptic plasticity [42,43]. PI3K is activated upon recruitment operated by the adaptor complex Shc-Grb2-Gab1. Otherwise, it can also be activated by Ras. PI3K activity leads to the activating phosphorylation of Akt, a serine-threonine kinase crucially involved in cell survival and growth [6]. For instance, Akt suppressed BAD activity, inhibiting the promotion of signaling cascades involved in apoptosis [44].

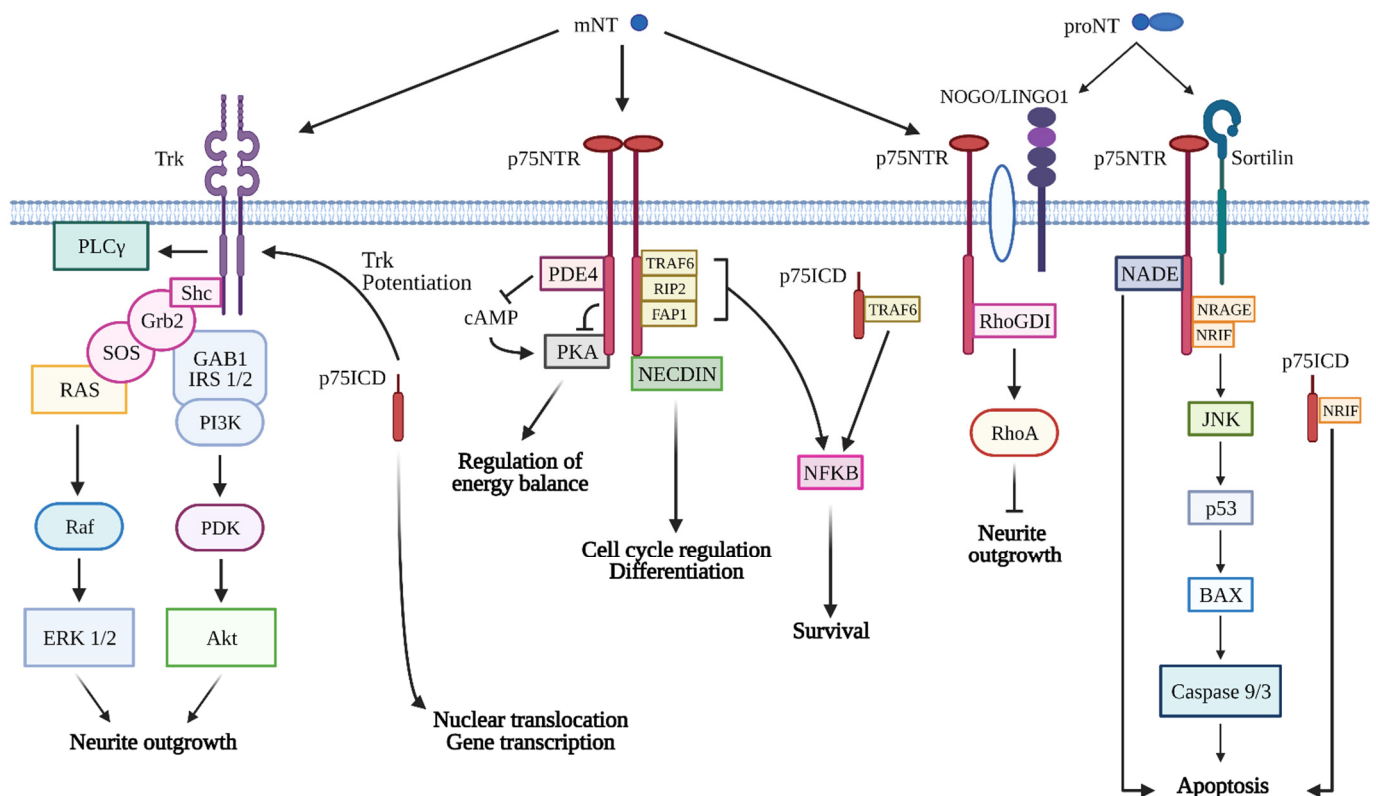


Figure 1. Signaling pathways mediated by neurotrophins' binding to their receptors. Neurotrophins bind to two main receptor types: Trk receptors and p75NTR. Pro-neurotrophins (proNT) processing generates mature neurotrophins (mNT). Upon mNT stimulation, Trk receptors undergo autophosphorylation events, and recruit adaptor proteins responsible for the activation of MAPKs, PI3K and PLC γ signaling cascades, involved in several neuronal functions such as neurite outgrowth. mNT may also bind to p75NTR, stimulating its proteolysis which releases the intracellular domain (p75ICD) that is essential for signalling and for the regulation of gene transcription. p75NTR activation by mNT may promote neuronal survival by inducing NF- κ B signaling. In addition, Necdin recruitment to p75NTR may regulate cell cycle progression and differentiation in a variety of cell types, whereas the interaction with PDE4 and PKA is involved in the regulation of energy balance. Importantly, in dependence on the cell context, p75NTR activation by mNT is able to potentiate the activation of Trk receptors. On the other hand, the binding of proNT to the p75NTR/Sortilin complex enhances the activation of cell-death-related pathways. When p75NTR interacts with NOGO/LINGO1, the recruitment of Rho GDI leads to the activation of RhoA, which in turn determines the inhibition of neurite outgrowth in neurons. FAP1, FAS-associated phosphatase 1; GAB1, GRB2 Associated Binding Protein 1; Grb2, growth factor receptor-bound protein 2; IRS 1/2, insulin receptor substrate 1/2; JNK, c-Jun N-terminal kinases; LINGO1, leucine-rich repeat and Immunoglobulin-like domain-containing protein 1; NADE, p75NTR-associated cell death executor; p75NTR, p75 neurotrophin receptor; NRAGE, neurotrophin receptor-interacting MAGE homologue; NRIF, neurotrophin receptor-interacting factor; PDE4, phosphodiesterase 4; PDK, phosphoinositide-dependent kinase; PKA, protein kinase A; PLC γ phospholipase C γ ; RhoA, Ras homolog family member A; RIP2, receptor-interacting protein 2; Shc, SRC homology domain-containing protein; SOS, son of sevenless; TRAF6, TNF receptor-associated factor 6; Trk, tropomyosin receptor kinase. This figure is created with BioRender.

Besides Trk receptors, NTs also induce their biological effects through p75NTR. P75NTR is a transmembrane glycoprotein receptor of approximately 75 kDa, belonging to the tumor necrosis factor (TNF) receptor family. P75NTR unselectively binds to all NTs with similar low affinity, whereas pro-NTs bind to p75NTR with high affinity [6]. The intracellular domain of p75NTR lacks catalytic activity, thus signaling pathways are mediated by the recruitment of adaptors and interacting proteins. p75NTR may form functional dimers, interacting with mature NTs. This interaction induces substantial conformational changes in the intracellular domain, leading to the recruitment of effector proteins [45]. TRAF6 is the most studied adaptor protein, and its activation is responsible for

NF- κ B-mediated survival [46,47]. NF- κ B activation may also be elicited by RIP2 recruitment [48]. Importantly, it has been recently shown that p75NTR-RIP2 interaction facilitates the survival of cerebellar granule neurons by promoting NF- κ B-dependent transcription of pro-survival genes [49,50]. p75NTR has also been reported to interact with Necdin. This MAGE-related protein together with E2F1, E2F4 and p53, represents a key regulator of the cell cycle, differentiation and survival in several tissues [7,51,52]. p75NTR activation is also implicated in the regulation of PKA signaling. Notably, phosphodiesterase PDE4A4/5 can be activated by p75NTR interaction, and this event leads to the increase of cAMP degradation and the subsequent PKA inactivation [53]. Furthermore, binding assays demonstrated that the intracellular domain of p75NTR interacts with both the catalytic and the regulatory PKA subunits. Hence, PKA activity may additionally be suppressed by direct interaction with p75NTR [54].

p75NTR not only forms homodimers, but also heterodimers with various receptors. These interactions frequently influence the binding to ligands and the consequent signaling pathways. For instance, pro-NTs have an increased affinity for p75NTR when receptor sortilin is co-expressed [55]. Pro-NTs-p75NTR-sortilin complex promotes the association of adaptor proteins involved in the induction of apoptotic cascades, including p75NTR-associated death executor (NADE), neurotrophin receptor-interacting factor (NRIF) and neurotrophin receptor-interacting MAGE homolog (NRAGE). These interactors mediate the stimulation of the c-Jun terminal kinases (JNK), which activate the transcription factor c-Jun and the tumor suppressor p53, resulting in the buildup of pro-apoptotic genes and, ultimately, cell death [47]. Specifically, it has been shown that p75NTR-dependent activation of p53 upregulates Bax expression and its mitochondrial translocation. These events result in cytochrome c release, caspase activation and neuronal apoptosis [56,57].

The formation of signaling complexes with Nogo and myelin-associated glycoprotein (MAG) facilitates the interaction of RhoGDI with the intracellular domain of p75NTR. Thus, p75NTR acts as a GDF displacement factor (GDF) that dissociates RhoA from RhoGDI. This event results in RhoA activation, which reflects in cytoskeleton rearrangement and inhibition of neurite outgrowth [58,59].

p75NTR is also often co-expressed with Trk receptors in diverse cell contexts. It has been reported that p75NTR interacts with TrkA [60], TrkB, and TrkC receptors [61], thereby modulating their affinity and response to NTs, and decreasing their endocytic internalization and degradation [62]. Notably, p75NTR potentiates the activation of Trk receptors, enhancing the survival and differentiation of neurons [63]. The molecular mechanisms by which p75NTR increases Trk activity are still not completely elucidated, and it was initially postulated that the two receptors may physically interact to form heterodimer complexes [60,61]. However, experimental findings demonstrated that there is no evidence for a direct interaction between p75NTR and TrkA extracellular domains, although the two receptors likely communicate through shared adaptor proteins or converging signaling pathways [64]. More recently, other studies highlighted that p75NTR and TrkA receptors are physically associated through the interaction of the intracellular domains, thus this complex can be formed independently of ligand stimulation [65].

Increasing evidence sustains that, similarly to Notch and amyloid precursor protein (APP), p75NTR is subjected to regulated intramembrane proteolysis, which generates soluble intracellular fragments capable of signaling properties. p75NTR is initially cleaved by the tumour necrosis factor- α converting enzyme (TACE/ADAM17) to yield a membrane-bound C-terminal fragment (CTF). Subsequently, CTF undergoes further cleavage by the γ -secretase, releasing the soluble intracellular domain (ICD) into the cytoplasmic compartment [66,67]. p75NTR ICD exerts several functions, ranging from survival to cell death, in relation to the co-receptors and/or the type of ligands [68]. In addition, recent reports indicated that p75NTR ICD may translocate to the nucleus, possibly regulating gene expression [7,68–70].

2.2. Neurotrophins in the Regulation of Cell Metabolism

Despite their critical role in the development, maintenance, survival and plasticity of the central nervous system, cumulative evidence indicates that NTs also as fundamental players in the regulation of metabolism and energy homeostasis. For this reason, these growth factors were designated as metabotrophins (from the greek *metabole*, and *trophe*, meaning “nutricious for metabolism”), highlighting the capability of NTs to regulate the activity of several cell processes, including those involved in glucose and lipid metabolism [71–73].

BDNF is the most extensively characterized NT concerning the control of energy homeostasis and mitochondrial metabolism. An interesting research study demonstrated that this growth factor affects energy metabolism in cortical developing neurons. BDNF upregulates the expression of the glucose transporter GLUT-3 and stimulates glucose utilization; a concurrent increase in amino acid uptake and protein synthesis are also observed upon BDNF administration: these events are essential to determine neuronal differentiation [74]. Signaling pathways mediated by BDNF through TrkB.T1/p75NTR interaction were shown to be determinants for the induction of glycogen synthesis in astrocytes. In contrast, when astrocytic p75NTR expression is reduced, the association between TrkB.T1 and adenosine receptor A_{2A}R or dopamine receptor D1R is favored, leading to the activation of PKA and subsequent induction of glycogen hydrolysis [75]. BDNF influences glucose metabolism not only in brain cells but also in peripheral tissues: recent findings showed that it exerts opposite effects on the transcripts of phosphoenolpyruvate carboxykinase (Pepck) and glycogen synthase kinase 3b (GSK3b) in hepatocytes, suggesting a reduction in gluconeogenesis and a concurrent enhancement in glycogen synthesis [76]. The impact of BDNF on systemic glucose metabolism is sustained by its crucial role in controlling the secretion of pancreatic hormones. Notably, β -cells express the TrkB.T1 receptor, and stimulation with BDNF triggers the release of calcium from the intracellular stores, enhancing insulin secretion induced by glucose [77]. On the other hand, pancreatic α -cells express the TrkB receptor and are responsive to BDNF, since its administration significantly reduces glucagon secretion [78]. In this context, it appears that BDNF may exert a general hypoglycemic effect by coordinating insulin and glucagon release. Coherently with this notion, it is becoming increasingly clear that BDNF signaling may participate in the development of type 2 diabetes mellitus (T2DM) [79–81]: specifically, BDNF levels were found to be decreased in patients with T2DM, and a negative correlation between insulin sensitivity and BDNF plasma concentration was also observed [82,83]. Interestingly, similar effects were also noted in rodent experimental models, where BDNF was shown to restore the alterations in pancreatic insulin and glucagon content in obese diabetic mice. In addition, BDNF administration also improved insulin resistance, body temperature and oxygen consumption in diabetic mice [84]. Another interesting report suggests that BDNF actions on diabetic hyperglycemia can be mediated, at least in part, by the effects induced in the brain, since intracerebroventricular or intra-ventromedial hypothalamic nucleus BDNF administration significantly counteracts hyperglycemia in streptozotocin-treated rats [85]. Additionally, recent data sustain that BDNF may exert a protective role against endothelial cell dysfunction observed in diabetic vascular complications, by activating mitophagy and by attenuating mitochondrial alterations and oxidative stress [86].

Energy metabolism is also influenced by NGF. Neuronal differentiation induced by NGF is accompanied by changes in ATP and NADPH levels, as well as a rise in oxygen consumption in rat pheochromocytoma PC12 cells. NGF-mediated mitophagy is also activated to assure the clearance of exhausted mitochondria, whereas the concurrent induction of mitochondrial remodeling and biogenesis sustains the energetic needs required during neuronal differentiation [87]. Similar to BDNF, experimental findings highlight a role for NGF in the hormonal control of glucose metabolism. Increased glucose levels stimulate the release of NGF and its signaling by TrkA receptors expressed by pancreatic β -cells, which in turn promotes F-actin rearrangement, granule mobilization and insulin

secretion [88]. Coherently with its hypoglycemic effect, NGF efficiently ameliorates the molecular alterations reminiscent of insulin resistance in basal forebrain cholinergic neurons [89]. In addition, NGF administration counteracts ER stress and apoptosis observed in Schwann cells during diabetic peripheral neuropathy, and efficiently attenuates neuronal death under glucose fluctuation in diabetic encephalopathy [90,91]. In contrast to BDNF and NGF, very little experimental evidence highlights the involvement of NT-3 or NT-4 in the regulation of energy homeostasis. However, NT-3 may exert important modulatory effects on mitochondrial dynamics increasing mitochondrial density along embryonic sensory axons [92]. The effects of NT-3 on cell energy metabolism were also studied in the pathological context of diabetes. Chiefly, NT-3 prevents the depolarization of the mitochondrial inner membrane in sensory neurons of streptozotocin-treated mice [93], and efficiently counteracts the elevation of blood glucose levels observed in obese diabetic mice [94]. The metabotropic activity of NTs is further corroborated by their modulatory role on skeletal muscle. Importantly, BDNF is expressed in skeletal muscle, and reprograms fiber metabolism by inducing a glycolytic phenotype [95]. Similar results were recently highlighted for NGF signaling: cell culture and *in vivo* experiments demonstrated that both satellite-cell-derived myoblasts and adult skeletal muscles express appreciable levels of p75NTR. Interestingly, skeletal muscle cells also synthesize NGF, with proNGF being the most predominant form. In this context, it has been reported that proNGF/p75NTR axis promotes the slow-to-fast fiber type transition by suppressing the slow/oxidative program, and by facilitating the induction of fast/glycolytic markers [7].

The regulation of cell metabolism by NTs is extended to the modulation of autophagy. Autophagy is a highly conserved biological process that is generally induced in response to nutrient starvation. It also mediates the degradation of macromolecules, and promotes the clearance of damaged organelles via the lysosomal pathway [96]. Under physiological conditions, basal autophagy flux is essential to maintain cellular homeostasis by participating in the routine turnover of cellular trash. Under stress and nutrient-deprivation, autophagy sustains cell survival by regenerating metabolites for the energy supply required during cell growth. Conversely, under pathological conditions, excessive induction of autophagy may be detrimental, leading to self-digestion and the degradation of key cellular components [96,97]. Most literature data point out a negative regulation on autophagy by BDNF [4]. For instance, BDNF signaling blocks autophagy in the forebrain of adult mice, and this event promotes synapse plasticity and memory [98]. Blockade of autophagy flux is also essential to guarantee the BDNF-mediated neuroprotective effects against mitochondrial dysfunction [96]. The mechanism by which BDNF suppresses autophagy seems to be ascribable to the activation of the TrkB signaling pathway. Specifically, it has been well-established that TrkB activation by BDNF elicits PI3K/akt/mTOR pathway, which is one of the most critical negative regulators of autophagy induction [15]. Similar to what was observed for BDNF, recent findings show that both NT-3 and NT-4 acts as autophagy inhibitors in different physiopathological conditions, such as colorectal cancers and functional recovery after spinal cord injury [99,100]. On the other hand, contradictory information is available concerning the involvement of NGF in autophagy modulation. Whereas some reports highlight that NGF inhibits autophagy [101,102], others provide evidence for NGF-mediated autophagy activation [103,104]. The nature of these conflicting results is not clear, however, it can be speculated that NGF may exert opposite roles on autophagy modulation, in dependence on the physiopathological context, the nutrient status of the cell, the relative abundance and the concurrent expression of TrkA/p75NTR receptors.

It has been well established that NTs may act as potent regulators of redox balance. NGF, BDNF and NT-4 exert neuroprotective effects by attenuating oxidative stress [105]. The antioxidant activity induced by these growth factors confers resistance to neuronal damage during neurodegenerative conditions, and is associated with the promotion of Nrf2 nuclear translocation and the subsequent heme oxygenase 1 (HO-1) upregulation [106–109]. The link between NTs and oxidative stress is further highlighted by the fact

that drinking habits and inadequate eating regimens can disrupt NTs metabolism, possibly by affecting ROS production. For instance, perinatal alcohol exposure, as well as chronic alcohol consumption during adulthood, is often associated with increased oxidative stress and altered NTs levels [110–112]. Interestingly, it has been observed that not only maternal but also paternal alcohol exposure can significantly impact NGF and BDNF levels in the brains of male offspring [113]. In addition, several lines of evidence indicate that polyphenols, powerful antioxidant compounds able to counteract oxidative damage induced by alcohol exposure, may elicit changes in NGF and BDNF expression as well as in their receptors [114–118].

Few studies suggest that NTs can also govern cell lipid metabolism. As an example, NGF-induced neuronal differentiation of PC12 cells is paralleled by changes in lipid metabolism [119]. Additionally, exercise can induce BDNF expression in skeletal muscle cells, which acts as a myokine to enhance fatty acid oxidation through AMPK activity [120]. Ultimately, p75NTR-influencing lipolysis and fat oxidation in adipocytes additionally support a part for NTs in the management of lipid homeostasis [54].

3. Neurotrophins and Cholesterol Metabolism

Increasing experimental evidence demonstrated that NTs exert important modulatory roles in several metabolic processes, and raised basic questions about their prospective involvement in the regulation of cholesterol metabolism. In this section, we will summarize and update the most outstanding discoveries underlying the complexity of cholesterol regulation in the whole body as well as in the brain compartment. Additionally, we will review the current knowledge on how NTs signalling may influence cholesterol homeostasis.

3.1. Overview of Cholesterol Homeostasis

Cholesterol is the most abundant sterol in animal tissues, and its homeostasis is essential for cell functioning and metabolism. It is mainly located on cell membranes, where it contributes to regulating the fluidity and the permeability of the phospholipidic bilayer; it is also involved in the modulation of membrane architecture and vesicle trafficking. Furthermore, cholesterol participates in the formation of specialized membrane microdomains known as lipid rafts [121,122].

In addition to its functions on the cell membrane, cholesterol is the precursor of bile acids, steroid hormones and vitamin D [5,123]. Alterations in cholesterol homeostasis are at the root of various congenital human diseases such as Niemann–Pick type C disease (NPC) [124,125] and Smith–Lemli–Opitz syndrome (SLOS) [126]. Additionally, increasing evidence suggests a tight relationship between alterations in cholesterol metabolism and different pathological conditions including many types of cancer [127–131], and neurodegenerative diseases such as Alzheimer’s disease (AD) [132–134], although the establishment of a direct connection is still unclear [135].

Proper intracellular cholesterol levels are maintained by the fine-tuning of de novo biosynthesis, uptake, efflux, and accumulation. From a systemic point of view, the regulation of cholesterol levels relies upon the interplay among several tissues and organs, which ensure homeostasis maintenance [136].

De novo synthesis is assured by the mevalonate (MVA) pathway which, starting from the acetate, leads to the production of cholesterol through ~ 30 enzymatic reactions. In humans, most cells can biosynthesize cholesterol. Notably, this biosynthetic pathway is particularly active in the intestines, muscles, and skin cells [137]. Nevertheless, about 50% of the total synthesis occurring in the whole body takes place in hepatocytes [138].

One of the most relevant proteins of this multi-enzymatic process is the 3 β -hydroxy-3 β -methylglutaryl CoA reductase (HMGCR). This Endoplasmic Reticulum (ER)-resident enzyme reduces the 3 β -hydroxy-3 β -methylglutaryl CoA (HMG-CoA) to MVA. HMGCR

represents the key and rate-limiting enzyme for cholesterol biosynthesis, thus its expression and activity are finely regulated at transcriptional, translational and post-translational levels [139].

Like other proteins involved in cholesterol homeostasis, HMGCR contains a sterol-sensing domain (SSD), which confers sensitivity to sterol levels in the ER membrane. In fact, when sterol levels are high, HMGCR interacts with the ER-resident protein insulin-induced gene (INSIG), thus undergoing ubiquitylation and subsequent proteasomal degradation [140].

Besides HMGCR degradation, long-term regulation of cholesterol metabolism is also operated by sterol regulatory element-binding proteins (SREBPs) transcription factors. Specifically, SREBP2 selectively regulates genes involved in cholesterol homeostasis [141,142]. When intracellular cholesterol levels decrease, SREBP2 is able to recognize and bind to specific DNA sequences called sterol regulatory elements (SREs), eliciting the transcription of several genes involved in cholesterol biosynthesis and uptake, including *hmgcr*, squalene monooxygenase (*sqle*) and low-density lipoprotein receptor (*ldlr*) [143].

SREBP2 is localized on the ER membrane, and its activation requires its transferring to the Golgi network. This event is mediated by the binding to SREBP cleavage activating proteins (SCAPs), which act as sterol sensors [144].

In the condition of sterol depletion, the SCAP/SREBP complex exits the ER and reaches the Golgi network, where SREBP undergoes proteolytic activation. Then, SREBP-derived N-terminal fragments are transcriptionally active and enter the nucleus to upregulate the expression of target genes [142]. Conversely, as intracellular sterol content rises, the SCAP/SREBP complex is restrained in the ER due to the interaction with INSIG proteins, thus preventing SREBP proteolysis and the subsequent gene transcription [144]. Even though the regulation of the SREBP pathway is well characterized, several modulatory mechanisms still remain poorly elucidated, and ongoing researches continuously shed light on novel players of cholesterol homeostasis. Four independent research groups recently demonstrated that the uncharacterized gene *C12orf49* is critically involved in the transcriptional control of cholesterol metabolism. Notably, *C12orf49* codifies for a glycosylated Golgi-resident membrane protein that is responsible for the proper SCAP expression and localization to the Golgi compartment. In addition, it has been demonstrated that site 1 protease (S1P) cleavage activity is promoted by the interaction with *C12orf49*, which in turn reflects in the proteolytic processing of several substrates, including SREBP [145–148].

Increasing evidence highlights that cholesterol biosynthesis is deeply subjected to epigenetic control [149]. For instance, very recent findings reported that the chromatin remodeling protein BRG1 is directly recruited to the SCAP promoter, influencing its transcription and the consequent SREBP maturation in liver cells [150]. The euchromatic histone-lysine N-methyltransferase 2 (EHMT2) acts as a cholesterol biosynthesis suppressor. EHMT2 inhibition by BIX01294 induces the expression of *sreb2* (encoding for SREBP2) through the reduction of H3K9me1 and H3K9me2 at the promoter [151]. Other reports also revealed that cholesterol biosynthesis may be strongly affected by the activity of bromo- and extra-terminal domain (BET) proteins, namely BRD2, BRD3, BRD4 and BRDT. Specifically, ChIP-seq and ChIP-qPCR analysis provided evidence that BRD4 is recruited to the promoters of a specific subset of genes, whose products belong to cholesterol biosynthetic pathway, such as farnesyl-diphosphate farnesyltransferase 1 (FDFT1), 24-dehydrocholesterol reductase (DHCR24), 7-dehydrocholesterol reductase (DHCR7) and mevalonate diphosphate decarboxylase (MVD). Importantly, BRD4 suppression by genetic knockdown or pharmacological inhibition efficiently displaces BRD4 from the promoters, thus reducing the expression of these target genes [152]. The epigenetic regulation of cholesterol biosynthesis by BET proteins is further sustained by another recent evidence, showing that BRD2 is recruited to the sigma 2 receptor (S2R), and cooperates with SREBP2 to regulate the subsequent expression of downstream targets involved in cholesterol metabolism [153]. Furthermore, BET inhibition by JQ1 significantly reduces the expression of

cholesterologenic proteins, such as SREBP2 and HMGCR, which reflects in a decreased intracellular cholesterol content [154].

Short-term regulation of cholesterol biosynthesis is mainly achieved by post-translational events that regulate HMGCR activity. Notably, the phosphorylation of a serine residue near the catalytic domain (Ser872 in humans) by AMP-activated protein kinase (AMPK) inhibits HMGCR activity. On the other hand, the dephosphorylation of the same residue, exerted by the protein phosphatase 2A (PP2A), reactivates the enzyme [155].

Even though an important source of cholesterol comes from endogenous biosynthesis, cholesterol intake from food sources represents about 22% of total plasma cholesterol [156]. Cholesterol absorption occurs on the apical surface of enterocytes in the intestinal lumen and on the bile canalicular membrane of hepatocytes, where the Niemann–Pick type C1-Like 1 protein (NPC1L1) is expressed [157]. Specifically, cholesterol interacts with the N-terminal domain of NPC1L1 [158]. Differently, the C-terminal domain presents an endocytic signal sequence YVNxxF to allow the internalization of NPC1L1; whereas a QKR sequence interacting with LIM domain and actin-binding protein 1 (LIMA1), is involved in the modulation of NPC1L1 trafficking [159,160]. NPC1L1 is localized in the endocytic recycling compartment (ERC) but, upon cholesterol depletion, the protein is exposed to the cell membrane [161,162]. Cholesterol binding to NPC1L1 leads to the dissociation of the NPC1L1 C-terminal tail from the cell membrane. This conformational change makes the endocytic motif accessible for the NUMB endocytic adaptor protein. NUMB activates the clathrin-mediated endocytosis by recruiting clathrin on invaginated microdomains, then the endocytic vesicles are internalized [159]. Subsequently, vesicles move along the actin filaments up to the ERC and NPC1L1 is recycled to the cell membrane for reuse [160,161,163,164].

Another well-known player involved in cholesterol metabolism is the low-density lipoprotein (LDL) receptor (LDLr), which mediates the uptake of cholesterol from the bloodstream [165]. This cell surface glycoprotein mediates the LDL endocytosis via its extracellular ligand-binding domain [166,167]. Cellular lipid uptake from LDL is ensured by the presence of apolipoproteins such as apoE and apoB100, which are incorporated into very-low-density lipoprotein (VLDL) and LDL, and are responsible for the binding to LDLr. Subsequently, the interaction of these proteins with their receptor leads to membrane invagination and endocytosis [168]. Due to the acid endosomal compartment, LDLr undergoes a conformational change and dissociates from LDL, and is eventually recycled back to the cell surface to start a new round of endocytosis [169–171].

Cholesteryl esters contained in LDL are hydrolyzed by lysosomal acid lipase to yield free cholesterol, which is exported from the lysosomal lumen and delivered to other cellular compartments via NPC1 and NPC2 [172,173]. Notably, mutations occurring on *npc1* or *npc2* genes determine an aberrant buildup of cholesterol into the lysosomal compartment, leading to the occurrence of a lysosomal lipid storage disorder known as NPC [174].

As already aforementioned, almost all mammalian cells can synthesize cholesterol. However, plenty of them are unable to efficiently catabolize it. Thus, cholesterol excess can leave the cell through efflux, or can be stored as cholesteryl-ester droplets.

Several proteins are involved in the efflux of cholesterol, such as ATP-binding cassette (ABC) subfamilies A and G [136] and extracellular apolipoproteins, which function as cholesterol acceptors [156]. ABCG5 and ABCG8 are almost exclusively expressed on the apical surface of hepatocytes and enterocytes: in this context, these transporters are involved in the excretion of cholesterol into the bile and the intestinal lumen, so that it can be eliminated by faeces [175].

The extracellular efflux does not constitute the only fashion through which mammalian cells can regulate the excessive concentration of intracellular cholesterol. Indeed, the production of cholesteryl esters, together with their storage in lipid droplets, represents another pivotal pathway that prevents free cholesterol accumulation. The Acyl-coenzymeA:Cholesterol Acyl-Transferase 1 (ACAT1) and ACAT2 isoenzymes are integral membrane proteins that take part in the esterification of cholesterol in different cell types

[176,177]. ACAT2 is mostly expressed in enterocytes and hepatocytes, and can be activated by the presence of the cholesterol itself to catalyze the esterification of different sterols or steroids containing a 3β -hydroxyl group with fatty Acyl-CoA [178–180]. The experimental data highlight that ACAT2 is more efficient in esterifying 25-hydroxycholesterol and derivatives from bile acids, but less efficient in esterifying cholesterol itself when compared to ACAT1 [181]. Interestingly, it has been demonstrated that ACAT2 activity is strongly associated with oxidative stress. In particular, the accumulation of sterol and fatty acids induces the production of reactive oxygen species (ROS) which oxidize ACAT2 at cysteine residue (Cys277), thus promoting the stabilization of the protein and enhancing storage and/or export of cholesterol [136].

3.2. Cholesterol Metabolism in the Brain

The central nervous system (CNS) represents the richest organ in cholesterol of the whole body, as almost 20% of this sterol is located in the brain [182]. The involvement of cholesterol in the CNS has been well established in many physiological processes by a plethora of studies. About 70–80% of the cholesterol in the adult brain can be partially found in the plasma membrane of neurons and astrocytes, where it influences cellular morphology, stabilizes cell surface receptors, and modulates synaptic transmission. However, the majority of this molecule is localized in myelin sheaths produced by oligodendrocytes, devoted to the insulation of the axons required for the saltatory impulse of the action potential [183]. Despite these roles, cholesterol is more than a simple structural component. Cholesterol is needed to form new membranes for axons or dendrites and for the development of pre-/postsynaptic spines [184–186]. Furthermore, it is also involved in axonal guidance regulation [187], synaptogenesis [188], microtubular transport of synaptic vesicles through the cytosol [189], and neurotransmitter release [168]. Cholesterol is also a pivotal constituent of lipid rafts, and its presence regulates the activity of membrane receptors such as γ -aminobutyric acid (GABA) receptor [190–192] or neurotrophin receptors (Trk and p75NTR) [193]. Considering its crucial role in the homeostasis maintenance in brain cells, it should not surprise that alterations of cholesterol metabolism are associated with a number of pathological conditions in the CNS. As a matter of fact, several neurodegenerative diseases and neurodevelopmental disorders are linked to alterations of cholesterol homeostasis, such as AD and Parkinson's disease (PD), NPC, Huntington's disease (HD) and Autism Spectrum Disorders (ASD) among others [194–200].

The blood–brain barrier (BBB) efficiently prevents the uptake of lipoproteins from the systemic circulation. Thus, brain cholesterol is produced by means of *de novo* synthesis *in situ*, which is finely regulated in diverse brain cells in dependence on the developmental stage [201]. It is widely accepted that during the embryogenesis—before astrocyte's differentiation—neurons autonomously synthesize cholesterol through the Kandutsch–Russel pathway [202,203]. However, during the postnatal period, neurons decrease or even interrupt their own synthesis whereas cholesterol production becomes particularly lively in astrocytes, through the Bloch Pathway [182,203,204]. Taken together, this evidence strengthens the hypothesis that mature neurons reduce or even abandon cholesterol synthesis to save the energy committed to the generation of the action potentials, thus relying on astrocytes to satisfy their cholesterol needs (Figure 2). Notably, experimental studies have highlighted that mice lacking cholesterol synthesis in adult neurons were phenotypically identical to controls, pointing out how cholesterol synthesis is not a crucial process in adult neurons [205]. In other words, the shutdown of cholesterol synthesis, counterbalanced by the increase in the intercellular trafficking of cholesterol from astrocytes via apoE-rich lipoproteins, ensures the ability to preserve energy in adult neurons for electrical activity. The export process begins in astrocytes, where newly-synthesized cholesterol is transferred by ABCA1 to extracellular lipid-free apoE [206]. Together with *apoE*, the *abca1* gene is expressed through the activation of Nuclear Liver X Receptors (LXRs). These transcriptional regulators sense the rising amount of cholesterol metabolites, such as cytosolic 24(S)-hydroxycholesterol (24-OHC) [182]. Through a feed-forward mechanism,

LXRs interact with 24-OHC and other oxysterols inducing the expression of the aforementioned genes [207].

The uptake of apoE-rich lipoproteins in neurons is then mediated by endocytosis via LDLr family members, i.e., LDLr and LDLr-related protein 1 (LRP1) [202]. In vitro studies have shown cholesterol in apoE-rich particles to increase the synaptic responses by improving presynaptic functions and dendrite differentiation [208,209]. Coherently with the neurotrophic effect of the intercellular cholesterol transport, LDLr or apoE deficiency severely impacts adult hippocampal neurogenesis [210,211]. ApoE is mainly produced by astrocytes, even though neurons are able to express apoE as well, in case of brain injuries [212]. Recently, it has been discovered that astrocyte-derived apoE increases histone acetylation in neurons, enhancing the transcription of immediate early genes involved in memory consolidation [213]. In humans, apoE is polymorphic, meaning it is present in three different codifying alleles: *apoE2*, *apoE3* and *apoE4*. ApoE4 polymorphism is one of the most well-studied examples of how cholesterol metabolism is linked to neurodegeneration. Specifically, apoE4 is likely to be involved in late-onset Alzheimer's disease, and is the most common risk-factor identified up to now for this pathology [214]: it affects amyloid- β aggregation and clearance [215,216], elicits synapse loss [217], and leads to the activation of microglia and astrocytes, thus causing neuroinflammation [218–220].

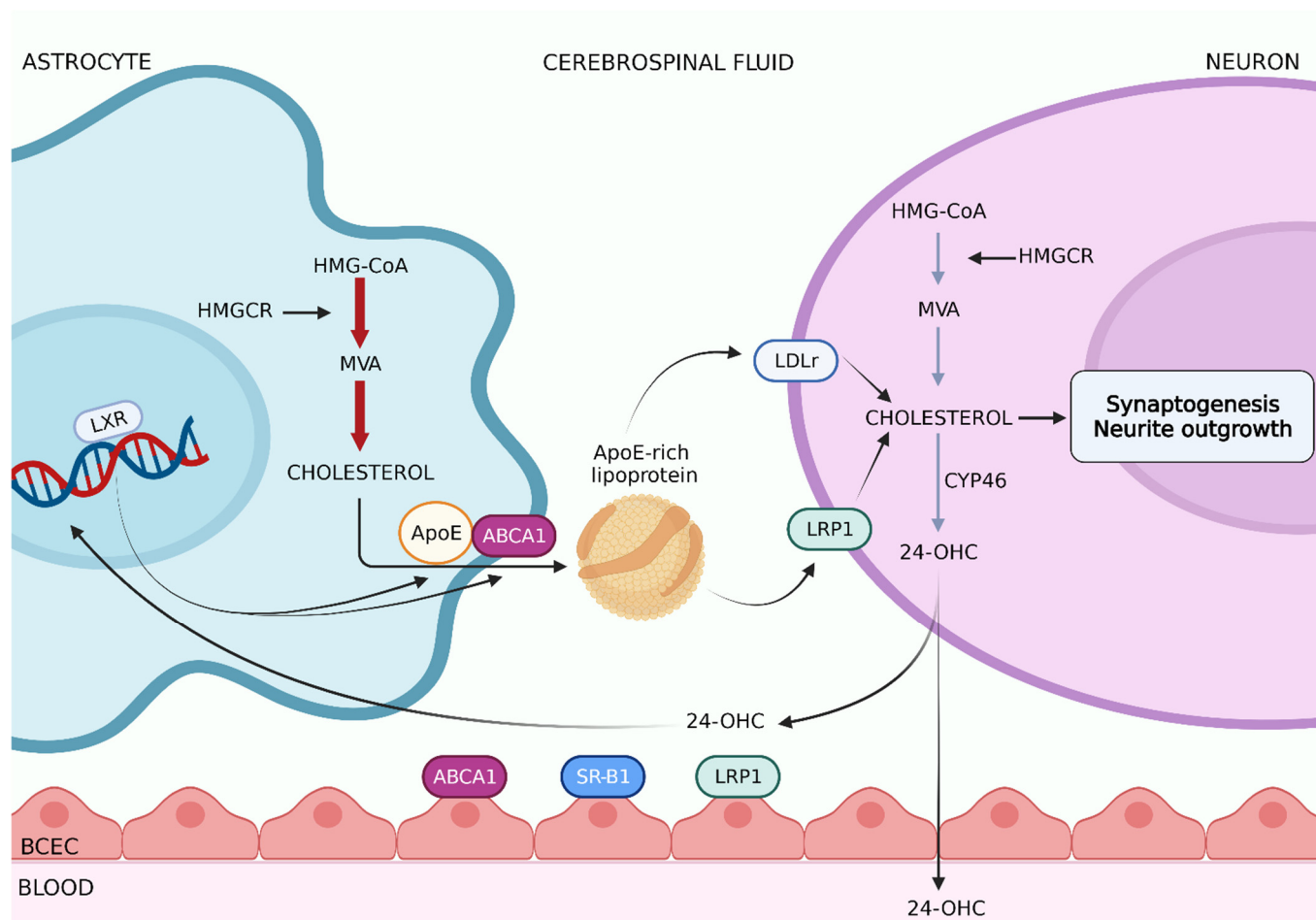


Figure 2. Cholesterol metabolism in the CNS. Cholesterol in the brain is assured by de novo biosynthesis requiring a multistep pathway. HMGCR is responsible for the conversion of HMG-CoA in MVA and represents the key and rate-limiting step. In the adult CNS, neurons reduce their own cholesterol synthesis and import this lipid from astrocytes, which secrete apoE rich-lipoproteins through ABCA1 transporters. ABCA1 and apoE transcription is elicited by LXR, whose activity is modulated by 24-OHC. Cholesterol uptake in neurons is ensured by LDLr and LRP1, particularly expressed on the cell membranes of neurons. Cholesterol excretion in the brain is mainly promoted by its conversion to 24-OHC catalyzed by CYP46. Abbreviations: 24-OHC, 24(S)-hydroxycholesterol; ABCA1, ATP-binding cassette transporter

A1; apoE, apolipoprotein E; BCEC, brain capillary endothelial cells; CYP46, cholesterol 24-hydroxylase; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; LDLR, LDL receptor; LRP1, LDLr-related protein 1; LXR, liver X receptor; MVA, mevalonate; SR-B1, scavenger receptor class B member 1. This figure is created with BioRender.

Once cholesterol-rich lipoproteins are endocytosed by the interaction between apoE and lipoprotein receptors, the apoE/cholesterol/LDLr complex is hydrolyzed, and free cholesterol is released and distributed through the different intracellular compartments [221] via NPC1 and NPC2, both expressed in neurons and glial cells [222].

As established before, due to its involvement in key neuronal processes, the amount of cholesterol in the brain must be finely controlled to ensure the correct brain functioning. Indeed, the presence of storage or secretion mechanisms is essential to counteract the putative excess of this sterol.

For instance, cholesterol in excess is esterified by ACATs enzymes mainly in the ER and stored in lipid droplets, representing about 1% of the total cholesterol content in the brain [223]. Despite ACATs are expressed in both neurons and glial cells, their activity is higher in neurons, where the esterification process strongly contributes to the maintenance of proper free cholesterol levels [224].

Besides esterification, the formation of oxysterols by hydroxylases represents the main way for cholesterol excretion from the brain. Cholesterol 24-hydroxylase is selectively expressed in the CNS, and is responsible for the conversion of cholesterol into its primary brain metabolite, 24-OHC: notably, 40% of cholesterol is released from the brain as 24-OHC [225]. This enzymatic conversion occurs in both neurons and glia cells, even though the major expression of cholesterol 24-hydroxylase takes place in neurons, especially in the pyramidal cells of the cortex and Purkinje cells of the cerebellum [226–228]. Oxysterols flow from the brain to the systemic circulation as a result of their capability to efficiently cross the BBB [229,230].

Another mechanism for cholesterol excretion is based on ABC transporters. In particular, ABCA1, ABCG1 and ABCG4 isoforms mediate cholesterol efflux from neurons to lipid-free apoA1 and High-Density Lipoprotein (HDL) particles [231]. Subsequently, secreted cholesterol-rich particles are released into the cerebrospinal fluid [232–234]. These lipoproteins also enter the systemic bloodstream by interacting with specific receptors such as LRP1 and scavenger receptor class B type I (SR-BI), both expressed in endothelial cells of brain capillaries [235,236].

Taken together, literature data highlight that cholesterol metabolism in the brain is controlled by intricate homeostatic mechanisms. In this context, neurons autonomously produce cholesterol during embryogenesis. Conversely, intercellular transport from astrocytes assures to neurons their cholesterol needs in the adult brain. Despite an adequate amount of cholesterol is essential to guarantee the proper activation of several neuronal processes, too much cholesterol may be detrimental. Thus, neurons manage the excess of cholesterol through different systems: the esterification, involving intracellular storage, or the excretion, using specific transporters. In addition, the conversion of cholesterol into more lipophilic molecules able to cross cell membranes (especially in the case of BBB), represents an important point of regulation to ensure that surplus cholesterol may efficiently exit the brain.

3.3. Regulation of Cholesterol Metabolism by Neurotrophins

As aforementioned, both cholesterol metabolism and neurotrophin pathways have been widely described as fundamental processes for the proper functioning of the whole body, including the CNS. A plethora of experimental evidence highlights that the presence of cholesterol in lipid rafts is essential to assure the correct translocation of neurotrophins' receptors on the plasma membrane, and its depletion disrupts the receptor functions and the downstream signaling cascades [237,238]. On the other hand, the role of NTs

in the modulation of cholesterol metabolism is still elusive and deserves further investigation. In this section, we will summarize the current knowledge proposing these growth factors as master regulators of cholesterol homeostasis (Table 1).

As previously reported, NGF is involved in the modulation of lipid metabolism. PC12 is a pheochromocytoma cell line derived from the peripheral nervous system (PNS) and it is able to assume a neuronal-like phenotype following appropriate stimuli. It has been observed that, upon NGF stimulation, these cells stop proliferating and undergo neurite extension. These evident morphological changes are accompanied by an increase in the small isoprenylated and methylated GTPases, highlighting the importance of protein prenylation in the acquisition of the neuronal-like phenotype induced by NGF. HMGCR activity is not only required for cholesterol production, but also for the synthesis of prenylated moieties employed for the post-translational modification and the subsequent attachment of small GTPases to cell membranes. In this context lovastatin, a powerful HMGCR inhibitor, reduces the amount of membrane-bound small GTPases, sustaining that NGF leads to an increase in the activity of the rate-limiting enzyme of the cholesterol biosynthetic pathway [239].

In addition to HMGCR, it has been reported that NGF may modulate the expression levels of other proteins involved in cholesterol metabolism, such as LRP1 and LDLr [240–242].

Following NGF administration, LRP1 membrane exposure and mRNA levels rapidly increase in GT1–1 Trk and PC12 cell lines. Interestingly, NGF treatment elicits the phosphorylation of the LRP1 cytoplasmic tail, and gives rise to the LRP1 endocytic activity [240]. More recently, NGF was also shown to activate the LRP1 promoter, further corroborating that NGF-mediated regulation of LRP1 occurs, at least in part, at the transcriptional level [241]. Taken together, these results confirm that NGF governs both short- and long-term regulation of LRP1 simultaneously with the neuronal differentiation process [240].

Other reports underlined that NGF increases LDLr expression levels in the pheochromocytoma cell line PC6.3 and in cultured septal neurons from embryonic rat brain, in a dose-dependent manner. The mature form of this neurotrophin induces *ldlr* gene transcription mainly through interaction with the TrkA receptor. LDLr upregulation is also promoted by pro-NGF binding to p75NTR receptor: coherently, it has been shown that p75NTR blockade selectively prevents the pro-NGF-induced increase in LDLr, whereas the upregulation of this receptor by mature NGF was suppressed upon TrkA inhibition. Similar to pro-NGF, pro-BDNF binding to p75NTR is also capable to enhance LDLr expression in PC6.3. Therefore, these data indicate that both mature NGF and pro-NGF influence LDLr expression by activating TrkA and p75NTR, respectively. In addition, the administration of both NGF and pro-NGF intensifies the uptake of lipoprotein particles. From a functional point of view, it has been revealed that the increased lipoprotein uptake promoted by NGF is a crucial mechanism that reinforces neurite outgrowth [242]. The effect of NGF on lipoprotein receptors is not delimited to LRP1 and LDLr, but also extends to apoER2. Differently from other LDLr family members, apoER2 only binds apoE-containing lipoproteins, and presents a high affinity for unrelated ligands such as reelin and clusterin. Besides mediation of macromolecules via endocytosis, apoER2 undergoes proteolytic cleavage and binds adapter proteins to activate different signal transduction pathways, most of them involved in brain development [243]. In this context, literature data elegantly illustrated that NGF triggers apoER2 proteolytic processing through TrkA-mediated signaling pathways [244]. NGF also influences cholesterol uptake by affecting the expression of apolipoproteins. For instance, it has been found that NGF treatment increases apoE transcript in PC12 cells, and this induction is attenuated by MAP kinase (MAPK) and PKC inhibitors, indicating that NGF-induced transcription of apoE requires the activation of TrkA/MAPK and TrkA/PKC axis [245].

Besides NGF, other experimental works pointed out a role for BDNF in the regulation of cholesterol metabolism. The results obtained by Suzuki and colleagues show that

BDNF, by interacting with the TrkB receptor, leads to a selective up-regulation of *hmgcr* and mevalonate pyrophosphate decarboxylase (*mpd*) transcripts in neurons, but not in glial cells [188]. This evidence was also confirmed by inhibiting HMGCR with mevastatin and squalene synthase with zaragozic acid, which efficiently prevents the BDNF-mediated increase in cholesterol biosynthesis. Interestingly, cholesterol enrichment after BDNF treatment is particularly visible in neuronal lipid rafts, and was paralleled by a concurrent increase in the expression of presynaptic proteins. In addition, electrophysiological approaches demonstrated that BDNF-mediated de novo cholesterol synthesis is involved in the generation of a readily releasable pool of synaptic vesicles [188].

Table 1. List of experimental evidence summarizing the role of neurotrophins in the modulation of cholesterol metabolism. ↑ indicate an increase; ↓ indicates a decrease.

Neurotrophin/Receptor	Cell Context	Effects	References
NGF	PC12 cell line	↑ HMGCR activity	[239]
NGF/TrkA	PC12 cell line	↑ apoER2 proteolytic processing	[244,245]
		↑ apoE transcription	
	GT1–1 Trk cell line; PC12 cell line	↑ LRP1 endocytic activity ↑ <i>LRP1</i> mRNA levels	[240,241]
NGF/TrkA pro-NGF/p75NTR pro-BDNF/p75NTR	PC6.3 cell line; Cultured septal neurons from embryonic rat brain	↑ LDLr expression ↑ Lipoprotein uptake	[242]
NGF/p75NTR	PC12 cell line	↑ Cholesterologenic enzymes	[246]
NGF/p75NTR pro-NGF/p75NTR	Huh7 hepatocyte cells	↑ Lipid- and cholesterol-associated proteins	[247,248]
BDNF/TrkB	Cerebral cortex; Hippocampal neurons from embryonic rats	↑ <i>hmgcr</i> and <i>mpd</i> transcripts	[188]
	NHA cells U87 MG cell line	↑ apoE biosynthesis; ↑ <i>ABCA1</i> levels; ↑ Cholesterol efflux;	[30]
	SH-SY5Y cell line	↑ <i>hmgcr</i> mRNA levels; ↓ Extracellular cholesterol uptake	
p75NTR	Neuro2a cell line; Primary cerebellar neuronal cultures	↑ <i>hmgcr</i> and <i>dhcr7</i>	[249]
NRIF/p75NTR	Neuro2a cell line	↑ Cholesterologenic enzymes	[250]

Other conclusions can be drawn from the results collected by Spagnuolo and collaborators, who recently highlighted the ability of BDNF to regulate apoE biosynthesis and the efflux of cholesterol from astrocytes, as well as its incorporation into neurons [30]. Specifically, BDNF stimulates cholesterol secretion from normal human astrocytes (NHA) and glioblastoma cell line U87 MG and, concurrently, increases ABCA1 expression levels.

These events are also accompanied by an increase in Erk1/2 phosphorylation [30]. It has been well-established that TrkB activation by BDNF upregulates the Erk1/2 signaling pathway [251] and regulates ABCA1 expression in different cell types [252,253]. Furthermore, the Erk1/2 pathway is involved in the increase of apoE expression and secretion by astrocytes [254]. Hence, these data support the hypothesis that BDNF-induced Erk1/2 activation may play a role in the modulation of apoE and ABCA1 in NHA and U87 MG glial cells.

In addition to cholesterol efflux from astrocytes, the authors revealed that BDNF administration increases *hmgcr* expression at mRNA level in the neuron-like SH-SY5Y cell line [30], further confirming the boosting effect of this neurotrophin on cholesterol biosynthesis in neurons [188]. On the other hand, BDNF significantly reduces the internalization of extracellular cholesterol and apoE interaction in the same neuronal cell line [30]. As already mentioned, the uptake of cholesterol is mediated by several receptors, including LDLr, VLDLr, apoER2 and LRP1 [255], widely expressed in the brain. The downregulation of these receptors is mediated by LXR-beta, a crucial regulator of cholesterol homeostasis in neurons, which increases following BDNF treatment: hence, BDNF reduces cholesterol uptake in neurons by raising LXR expression [30]. Collectively, these results highlight that BDNF orchestrates a complex homeostatic regulation, by enhancing the biosynthesis of cholesterol, and by suppressing its uptake in neurons. The reduction of extracellular cholesterol uptake in neurons may be attributable to the important neuroprotective role of BDNF. Indeed, if on one hand proper cholesterol levels are indispensable for assuring the correct induction of key neuronal processes, an excess of cholesterol in the brain can be toxic, causing apoptosis [256–259] and contributing to the formation and deposit of the beta-amyloid peptide [260].

The crucial role of neurotrophins in the regulation of cholesterol metabolism is further strengthened by few studies, which specifically investigated the involvement of the pan-neurotrophin receptor p75NTR. p75NTR appears to be a potent regulator of the enzymes involved in cholesterol biosynthesis in neuronal cultures and in various neuroblastoma cell lines. Notably, a relationship between p75NTR and cholesterologenic enzymes has been demonstrated by comparing p75NTR-positive and p75NTR-negative PC12 cells. These cells were treated with neocarzinostatin (NCS), an antimitotic agent capable of inducing cell death. Interestingly, the susceptibility to NCS was higher in p75NTR-positive cells than in p75NTR-negative cells. p75NTR expression was associated with an upregulation of enzymes responsible for cholesterol biosynthesis, such as HMGCR, farnesyl-diphosphate synthase, and 7-dehydro-cholesterol reductase. In order to understand whether the pro-apoptotic effects of p75NTR depended on increased cholesterol biogenesis, the authors used mevastatin to inhibit HMGCR. Upon mevastatin administration, NCS susceptibility between p75NTR-positive and p75NTR-negative cells was similar, indicating that cholesterol biosynthesis contributes to potentiate the pro-apoptotic effects of p75NTR [246]. The dependence of cholesterol biosynthesis on p75NTR activity is further sustained by Korade and colleagues. Even though the reduction in cholesterol biosynthesis in adult neurons is a widely accepted model, some neuron types may retain the capability to synthesize cholesterol in the adult brain, as the cholesterol biosynthetic enzymes HMGCR and DHCR7 are co-localized in some cortical, hippocampal and cholinergic neurons in the adult mouse brain. Considering that hippocampal and cholinergic neurons express p75NTR, the authors hypothesized that p75NTR could modulate the expression levels of enzymes belonging to the cholesterol biosynthetic pathway. Indeed, they silenced p75NTR expression in Neuro2a cells and in primary cerebellar neuronal cultures, and found that p75NTR suppression determined a significant decrease in the expression of HMGCR and DHCR7, possibly via the SREBPs-dependent pathway [249].

Successively, it has been found that the effects of p75NTR on the regulation of cholesterol and lipid biosynthetic genes are mediated by NR1F, both *in vitro* and *in vivo*. In fact, these findings imply that the NR1F-regulated endogenous cholesterol biosynthesis is

crucial for neuronal homeostasis and that the addition of exogenous cholesterol cannot prevent the harmful effects derived from an altered biosynthesis [250].

Recently, it has been shown that p75NTR, in response to NGF or pro-NGF, is able to modulate the expression of LDLr in Huh7 hepatocytes, by inducing SREBP2 cleavage. Signal cascades leading to SREBP2 cleavage include the activation of p38 MAPK and caspase-3: p38 MAPK increases caspase-2 phosphorylation, thus reducing its interaction with caspase-3. Caspase-3, for its part, determines the cleavage of SREBP2, which exhibits a caspase-3 cleavage site. Then, SREBP2 induces the transcription of *ldlr* and other lipogenic genes. Thereby, the SREBP2 pathway mediated by the neurotrophin/p75NTR system differs from that induced by sterol deficiency, even though they could functionally interact under stress conditions and in case of abnormal lipid accumulation [247,248].

4. Conclusions

Even though initially identified as specific neurotrophic factors selectively committed to the survival and the differentiation of neuronal cells, compelling evidence highlights the crucial role of NTs in the regulation of cell metabolism in different cell contexts. The current knowledge summarized in this review underlines the crucial involvement of NTs in metabolic processes related to the control of glucose homeostasis, autophagy, oxidative stress, energy expenditure and lipid metabolism. Notably, some members belonging to the NT family also show important modulatory activities on cholesterol metabolism. When evaluated as a whole, literature data seem to suggest that, independently of the cell context, NGF and BDNF may promote an overall activation of cholesterol biosynthesis in different cell types [188,239,248]. Conversely, the effect on cholesterol uptake can be differently modulated in neuronal cells: specifically, BDNF suppresses cholesterol uptake [30], whereas NGF stimulates the internalization of lipoprotein particles [242]. This discrepancy can be explained by considering the different experimental setups, and the different roles potentially exerted by the two NTs. Indeed, cholesterol requirements are strongly increased to sustain neuritogenesis and synaptogenesis: thus, NGF may promote neuronal differentiation by enhancing LDL uptake to support the formation of new membranes. On the contrary, the reduction of cholesterol uptake induced by BDNF in already differentiated neuronal cells may represent a protective mechanism to prevent the toxicity induced by excessive cholesterol. Despite some hints on how NTs regulate cholesterol metabolism are now available, some information is still lacking, and a number of unanswered questions deserve further investigation. For instance, unlike BDNF, there are no systematic studies aimed at evaluating the prospective role of NGF in the regulation of cholesterol metabolism in astrocytic cells. Considering that neurons depend on cholesterol produced by astrocytes in the adult brain, it could be fruitful to comprehend whether NGF is able to affect the intercellular cholesterol transport, whose proper regulation is indispensable to guarantee brain physiology. Furthermore, the role of NTs other than NGF and BDNF, such as NT-3 and NT-4, is completely missing in the context of cholesterol homeostasis. A deeper comprehension of the molecular aspects linking NTs and cholesterol metabolism may be useful to better contextualize the involvement of NTs in different brain disorders and, more extensively, in a plethora of pathological conditions characterized by the loss of cholesterol homeostasis.

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NGF Modulates Cholesterol Metabolism and Stimulates ApoE Secretion in Glial Cells Conferring Neuroprotection against Oxidative Stress

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Abstract: Cholesterol plays a crucial role in the brain, where its metabolism is particularly regulated by astrocytic activity. Indeed, adult neurons suppress their own cholesterol biosynthesis and import this sterol through ApoE-rich particles secreted from astrocytes. Recent evidence suggests that nerve growth factor (NGF) may exert neurotrophic activity by influencing cell metabolism. Nevertheless, the effect of NGF on glial cholesterol homeostasis has still not been elucidated. Thus, the aim of this project is to assess whether NGF could influence cholesterol metabolism in glial cells. To reach this objective, the U373 astrocyte-derived cell line was used as an experimental model. Immunoblot and ELISA analysis showed that proteins and enzymes belonging to the cholesterol metabolism network were increased upon NGF treatment in glial cells. Furthermore, NGF significantly increased ApoE secretion and the amount of extracellular cholesterol in the culture medium. Co-culture and U373-conditioned medium experiments demonstrated that NGF treatment efficiently counteracted rotenone-mediated cytotoxicity in N1E-115 neuronal cells. Conversely, neuroprotection mediated by NGF treatment was suppressed when N1E-115 were co-cultured with ApoE-silenced U373 cells. Taken together, these data suggest that NGF controls cholesterol homeostasis in glial cells. More importantly, NGF exerts neuroprotection against oxidative stress, which is likely associated with the induction of glial ApoE secretion.

Keywords: ApoE; astrocyte; cholesterol; differentiation; nerve growth factor; metabolism; neuron; reactive oxygen species; rotenone



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1. Introduction

The brain is characterized by the highest amount of cholesterol in the whole body [1]. The exceptional concentration of this sterol in the central nervous system (CNS) is justified by the fact that cholesterol is involved in several fundamental biological processes. Of note, a significant amount of cholesterol is integrated into plasma membranes of astrocytes and neurons, where it participates in defining a proper cellular morphology, as well as the correct functioning of membrane receptors and synaptic neurotransmission [2]. Cholesterol is also essential to form new membranes required for neurite outgrowth, synaptogenesis, and neurotransmitter release, thus exerting a crucial role in neuronal differentiation and maturation [3–7].

Essentially, all cholesterol in the brain derives from de novo biosynthesis in situ. Specifically, the blood brain barrier (BBB) prevents cholesterol uptake deriving from lipoproteins transported through the systemic circulation [8]. For this reason, nerve cells develop a fine

regulatory network to independently manage their own demand for this lipid. A well-accepted model establishes that after embryogenesis, during the postnatal period, cholesterol biosynthesis dramatically drops in neurons, with the attempt to save the energy employed for the generation of the action potentials. Accordingly, astrocytes satisfy neuronal requirements of cholesterol, whose synthesis becomes particularly pronounced [1,9,10]. The delivery of cholesterol produced by astrocytes to neurons implies an elegant mechanism of horizontal transport, which involves key proteins and particles. Specifically, cholesterol export starts in astrocytes, where neo-synthesized cholesterol is loaded into nascent lipoprotein particles containing apolipoprotein E (ApoE). Accumulating evidence indicates that the lipidation of ApoE-rich particles is mediated by the transporter ATP-binding cassette A1 (ABCA1) [11]. Subsequently, once released into the extracellular space, ApoE-containing lipoproteins are up taken by neurons through receptor-mediated endocytosis via low density lipoprotein receptor (LDLr) and LDLr-related protein 1 (LRP1) [12]. According to the pivotal role of astrocyte-derived cholesterol in neuronal homeostasis, experimental findings indicated that cholesterol contained in ApoE-rich lipoproteins significantly enhances dendrite differentiation and synaptic function [13,14]. Accordingly, astrocyte-conditioned medium from Huntington's disease (HD) astrocytes, which only secrete a modest amount of ApoE-lipoprotein-bound cholesterol, severely affects neurite outgrowth and synaptic activity in HD neurons [15]. The neurotrophic action of astrocyte-derived ApoE-containing particles is further sustained by the impact of ApoE deficiency, which markedly hampers adult neurogenesis in the hippocampus [16]. Astrocyte–neuron cholesterol crosstalk is also critical in neuroprotection against oxidative stress. For instance, some reports show that ApoE hinders oxidative damage and apoptosis induced by hydrogen peroxide exposure [17,18].

From these observations, the maintenance of cholesterol homeostasis in the brain is necessary to guarantee the functioning of nerve cells, and the metabolic pathways underlying the regulation of this lipid should be finely controlled to preserve the occurrence of several neurophysiological processes.

In recent years, neurotrophins have emerged as important players in cell metabolism [2,19–22]. Neurotrophins belong to a small family of growth factors that have an incontrovertible role in neuronal development, survival, and plasticity [23]. More recently, these signaling molecules have also been recognized as crucial modulators of cell metabolism, including cholesterol metabolism [2,22].

Experimental evidence highlights that nerve growth factor (NGF), the prototype member of the neurotrophin family, stimulates cholesterol biosynthesis in neuronal cells and hepatocytes [24,25]. Interestingly, these events involve, at least in part, the low-affinity neurotrophin receptor p75NTR, which elicits activation of the sterol regulatory element binding protein 2 (SREBP-2) and, in turn, induces the transcription of cholesterol-related genes [25]. The activity of NGF on cholesterol metabolism is not restricted to biosynthesis, as it also promotes cholesterol uptake by increasing LDLr expression in neurons [26].

Despite this information, knowledge about the possible contribution of NGF in the regulation of cholesterol homeostasis still appears particularly limited. Notably, there is a lack of systematic studies assessing the role played by NGF in the regulation of cholesterol metabolism in astrocytic cells. Considering that cholesterol produced by astrocytes is essential to guarantee neuronal physiology, it is important to understand whether NGF is able to influence cholesterol metabolism in glial cells as well as intercellular cholesterol transport, whose peculiar regulation is imperative to assure brain health.

On this basis, the aim of this work is to explore the prospective involvement of NGF in the modulation of cholesterol metabolism in glial cells, as well as its impact on neuronal differentiation and neuronal survival under oxidative stress conditions.

2. Results

2.1. U373 Express Both the NGF Receptors TrkA and p75NTR

NGF was initially identified as a growth factor with a specific tropism for neuronal cells. However, accumulating evidence clearly demonstrate that this neurotrophin also

induces biological effects in several non-neuronal cells and tissues [22]. The activity of NGF is assured by its binding to the high-affinity receptor TrkA and to the low-affinity receptor p75NTR [2]. Thus, we initially ascertained whether U373 cells express NGF receptors. Notably, U373 is an astrocytoma cell line widely employed as an astrocyte cell culture model, as several reports indicated that they reproduce a number of morphological, metabolic, and functional properties of astrocytes [27–29]. According to published data [30], immunofluorescence analysis showed that U373 cells express both TrkA and p75NTR at appreciable levels (Figure S1A). These results were also confirmed by Western blot analysis, suggesting that U373 cells have the potential to be fully responsive to NGF. Since the administration of NGF may affect the expression levels of its receptors, potentially altering the balance between signaling pathways elicited by TrkA and/or p75NTR [31–33], the expression of both receptors was also checked upon 48 h of NGF treatment. However, no changes were observed in the protein amount of both TrkA and p75NTR after the stimulation with the neurotrophin (Figure S1B).

2.2. NGF Modulates the Protein Network Involved in Cholesterol Homeostasis in Glial Cells

Once ascertained that U373 cells are responsive to NGF, we evaluated the putative role of NGF in the modulation of the main proteins belonging to cholesterol homeostasis machinery. We started to check SREBP-1 and SREBP-2, the most relevant transcription factors of lipid-related genes. In particular, when intracellular lipids are low, SREBPs are activated by proteolytic cleavage, generating a transcriptionally-active fragment that enters the nucleus and induces the transcription of target genes [2]. Immunoblot analysis revealed that NGF treatment did not affect the expression of the full length and the nuclear form of SREBP-1 (Figure 1A). Conversely, stimulation with the neurotrophin significantly increased both the full length and the active fraction of SREBP-2 (Figure 1B). We next evaluated HMGCR expression, the key and rate-limiting enzyme of cholesterol biosynthesis, revealing a build-up of this enzyme following NGF treatment (Figure 1C). While LDLr was not affected (Figure 1D), NGF treatment increased NPC1, which is involved in intracellular cholesterol trafficking (Figure 1E). In addition, NGF significantly raised ABCA1 levels (Figure 1F), the most important transporter involved in cholesterol efflux from astrocytes [2], whereas intracellular ApoE expression did not show any variation. Interestingly, the ELISA assay displayed a significant ApoE enrichment in U373 conditioned medium upon NGF exposure (Figure 1H). Taken together, these results indicate that NGF markedly impacts cholesterol metabolism in glial cells by increasing the expression of proteins involved in cholesterol biosynthesis, trafficking, and efflux. Of note, the induction of ABCA1 expression strongly suggests an enhancement in the secretion of ApoE-rich particles from astrocytes, which is corroborated by the rise in ApoE levels into the culture medium.

2.3. NGF Increases Cholesterol Secretion by U373 into the Culture Medium

The obtained results prompted us to study whether the effects mediated by NGF could reflect the modulation of cholesterol content. Oil Red O staining was performed to visualize neutral lipids, including cholesteryl esters. The size and the number of lipid droplets found in U373 cells were similar to those ones reported for primary astrocytes [34,35]. However, no alterations were observed upon NGF treatment (Figures 2A and S2A,B). Similarly, no changes were detected in the amount of free cholesterol, evaluated by filipin staining (Figures 2B and S2C). The lack of significant effects on intracellular cholesterol levels was further sustained by cholesterol enzymatic assay, which highlighted that NGF did not alter the content of total cholesterol, free cholesterol, and cholesteryl esters (Figure 2C). Conversely, the amount of cholesterol (free, esters, and total) was markedly increased in the culture medium of U373-treated NGF (Figure 2D). When evaluated as a whole, these data indicate that NGF promotes cholesterol extrusion by enhancing ABCA1-mediated efflux of ApoE-containing particles.

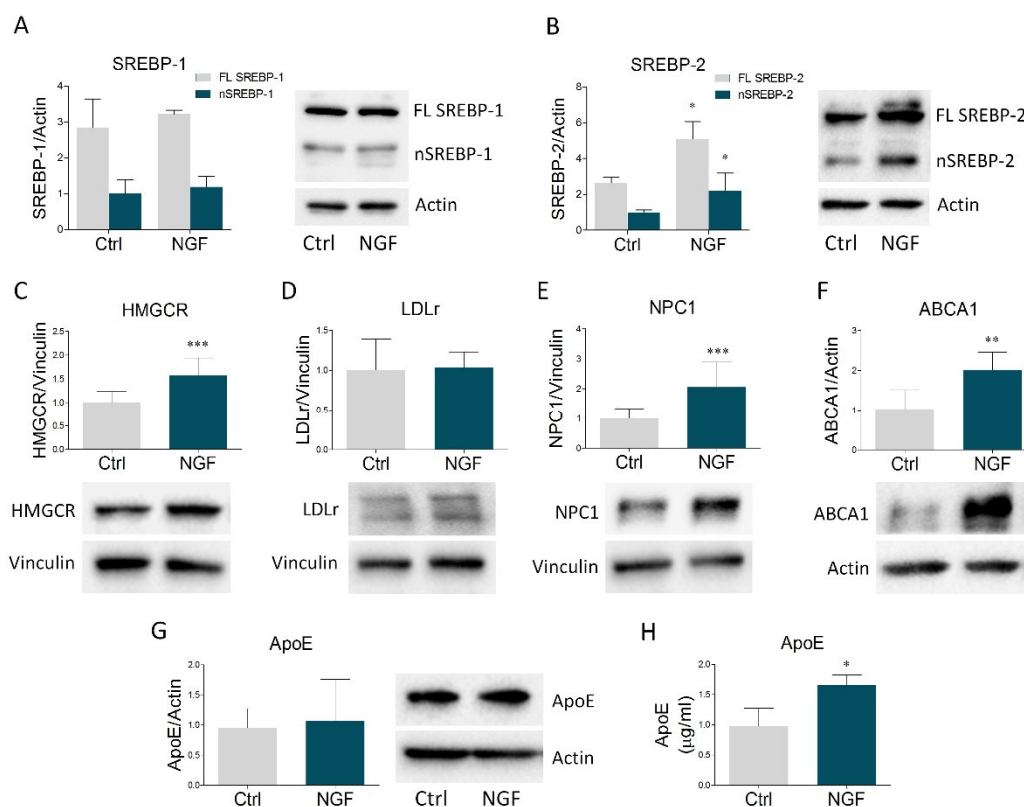


Figure 1. NGF modulates the expression of the main proteins involved in cholesterol homeostasis in U373 cells and enhances ApoE secretion in U373 culture medium. (A–G) Representative Western blot and densitometric analysis of SREBP-1, SREBP-2, HMGCR, LDLr, NPC1, ABCA1, and ApoE in U373 cells treated with vehicle (Ctrl) and NGF (100 ng/mL) for 48 h. $n = 6$ different experiments. Actin and vinculin were used as loading controls. (H) Quantification of ApoE levels ($\mu\text{g/mL}$) by ELISA assay in vehicle- and NGF-treated U373 culture medium. $n = 3$ different experiments. Data represent means $\pm$ SD. Statistical analysis was assessed by using unpaired Student's t test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.4. p75NTR Is Partially Involved in the NGF-Mediated Effects on Cholesterol Protein Network

Experimental findings show that NGF affects cholesterol metabolism in liver cells through the activation of its low-affinity receptor p75NTR [25]. To test whether this signaling pathway may be also preserved in astrocytic cells, we treated U373 with LM11A-31, a small ligand acting as a specific p75NTR modulator. This compound does not activate TrkA but, similarly to NGF, elicits the induction of p75NTR downstream signaling pathways [36]. LM11A-31 mimicked the NGF-mediated increase of HMGCR (Figure 3A). However, no changes were observed in the other proteins involved in cholesterol metabolism (Figure 3B–D), nor in the amount of extracellular ApoE (Figure 3E) and cholesterol (total, free and esters) released into the culture medium (Figure 3F). Collectively, these results indicate that NGF-induced expression of HMGCR is mediated by p75NTR activity, whereas the effects on NPC1, ABCA1, ApoE secretion, and cholesterol extrusion are likely dependent on other transduction pathways, possibly involving TrkA.

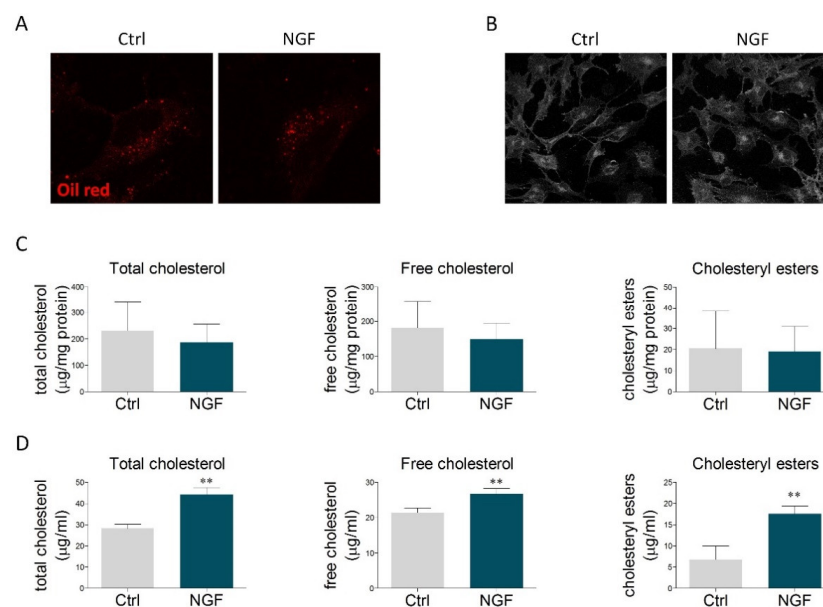


Figure 2. NGF effects on neutral lipids and cholesterol content. (A) U373 cells were treated with vehicle (Ctrl) or NGF (100 ng/mL) for 48 h and then stained with Oil Red O to visualize the intracellular content of neutral lipids. $n = 3$ different experiments. (B) Representative image of filipin staining performed on U373 cells treated with vehicle (Ctrl) and NGF (100 ng/mL) for 48 h. $n = 3$ different experiments. (C) Quantification of intracellular cholesterol levels (total cholesterol, free cholesterol, and cholesteryl esters) in vehicle- and NGF-treated U373 cells. $n = 3$ different experiments. (D) Cholesterol quantification (total cholesterol, free cholesterol, and cholesteryl esters) in the culture medium of U373 cells treated with vehicle (Ctrl) and NGF (100 ng/mL) for 48 h. $n = 3$ different experiments. Data represent means $\pm$ SD. Statistical analysis was performed by using unpaired Student's t -test. ** $p < 0.01$.

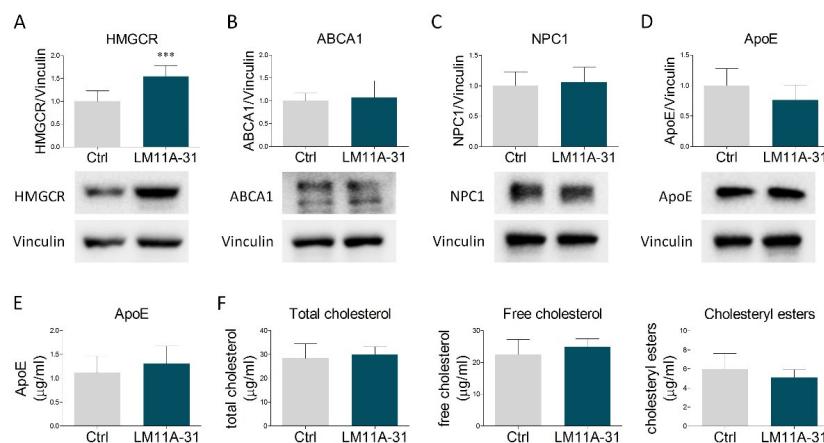


Figure 3. Effects of p75NTR modulation by LM11A-31 on cholesterol metabolism and secretion. (A–D) Representative Western blot and densitometric analysis of HMGCR, ABCA1, NPC1, and ApoE in U373 cells treated with vehicle (Ctrl) and LM11A-31 (0.1 µM) for 48 h. $n = 4$ different experiments. Vinculin served as a housekeeping protein to normalize protein loading. (E) Quantification of ApoE amount (µg/mL) by ELISA assay in culture medium from vehicle- and LM11A-31-treated U373 cells. $n = 3$ different experiments. (F) Cholesterol quantification (total cholesterol, free cholesterol, and cholesteryl esters) in the culture medium of U373 cells treated with vehicle (Ctrl) and LM11A-31 (0.1 µM) for 48 h. $n = 3$ different experiments. Data represent means $\pm$ SD. Statistical analysis was carried out by using unpaired Student's t test. *** $p < 0.001$.

2.5. Conditioned Medium Derived from NGF-Treated U373 Cells Does Not Influence Neuronal Differentiation

Published data demonstrate that astrocyte-derived ApoE particles could impact neurite outgrowth in neurons [15]. Thus, we evaluated whether NGF-mediated secretion of ApoE can affect neuritogenesis in the neuron-like cell line N1E-115. To reach this aim, N1E-115 were induced to differentiate for 48 h in the presence of conditioned medium derived from control U373 (U373-Ctrl), NGF-pre-treated U373 (U373-NGF), and NGF-pre-treated U373 silenced for ApoE (U373-NGF ApoE siRNA). The obtained results showed that no effects were detectable in the percentage of neurite-bearing cells, neurite length and number of neurites among the experimental groups taken into consideration in this study (Figure 4A,B). Therefore, U373-conditioned medium and/or U373-derived ApoE do not influence N1E-115 neuritogenesis, even when U373 are pre-treated with NGF.

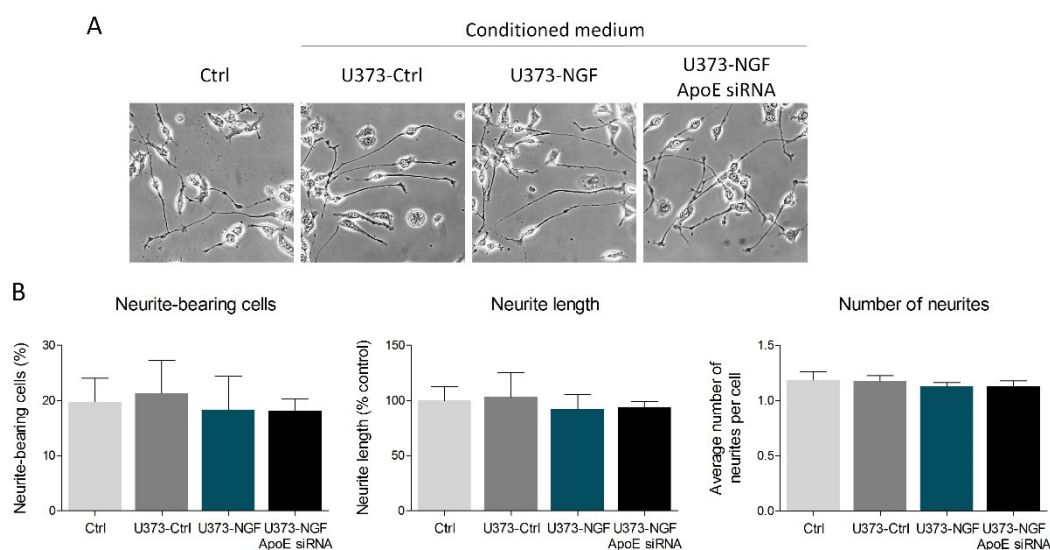


Figure 4. Effect of U373 conditioned medium on neuronal differentiation. (A) Representative images in bright field of N1E-115 cells cultured in fresh DMEM (Ctrl), in conditioned medium derived from control U373 (U373-Ctrl), NGF-pre-treated U373 (U373-NGF), and NGF-pre-treated U373 silenced for ApoE (U373-NGF ApoE siRNA) for 48 h. (B) Morphological analysis of neurite-bearing cells, neurite length and number of neurites of N1E-115 cells in the presence of fresh DMEM (Ctrl), in conditioned medium derived from control U373 (U373-Ctrl), NGF-pre-treated U373 (U373-NGF), and NGF-pre-treated U373 silenced for ApoE (U373-NGF ApoE siRNA) for 48 h. $n = 5$ different experiments. Data represent means $\pm$ SD. Statistical analysis was performed by using one-way ANOVA, followed by Tukey's post hoc test.

2.6. NGF-Mediated Secretion of ApoE by U373 Protects Neuronal Cells from Oxidative Insult

Accumulating evidence highlights that ApoE increases neuronal resilience to oxidative insults, thus preventing apoptosis and neurodegeneration [17,18,37]. In this context, we evaluated whether NGF-induced ApoE secretion by U373 cells could protect N1E-115 neurons from oxidative stress. Fully differentiated N1E-115 were pre-treated with rotenone, which induces oxidative stress by inhibiting the mitochondrial complex I. After 16 h of rotenone exposure, N1E-115 neurons were cultured in fresh medium (Ctrl), conditioned medium from U373 cells (U373-Ctrl), conditioned medium from U373 pre-treated with NGF for 48 h (U373-NGF), or conditioned medium from ApoE-silenced U373 pre-treated with NGF for 48 h (U373-NGF ApoE siRNA). Rotenone pre-treatment strongly decreased the number of N1E-115 cultured in control medium, indicating that the administration of this pesticide severely impacts N1E-115 survival. Rotenone cytotoxicity also determined intense neurite retraction, as observed by the length of neurites. Neurite damage elicited by rotenone treatment was particularly evident upon evaluation of neurite-bearing cells, as the number of N1E-115 with neurites decreased to 50%. Similar results were obtained

when N1E-115 cells were cultured in U373-Ctrl conditioned medium, suggesting that the presence of supernatant derived from unstimulated astrocytes is not sufficient to protect neuronal cells from rotenone-mediated toxicity. On the contrary, application of conditioned medium derived from NGF-treated U373 effectively prevented neuronal death, as well as the reduction in neurite-bearing cells and neurite length caused by rotenone administration (Figure 5). More importantly, conditioned medium derived from ApoE-silenced U373 cells completely lost the beneficial effects induced by U373-NGF conditioned medium, indicating that neuroprotection is driven by the NGF-mediated ApoE release in the culture medium. Notably, conditioned medium from NGF-treated U373 transfected with scrambled siRNA fully retained the protective effects exerted by U373-NGF conditioned medium, demonstrating that the loss of neuroprotection observed in conditioned medium from ApoE-silenced cells is effectively dependent upon ApoE downregulation (Figure S3C).

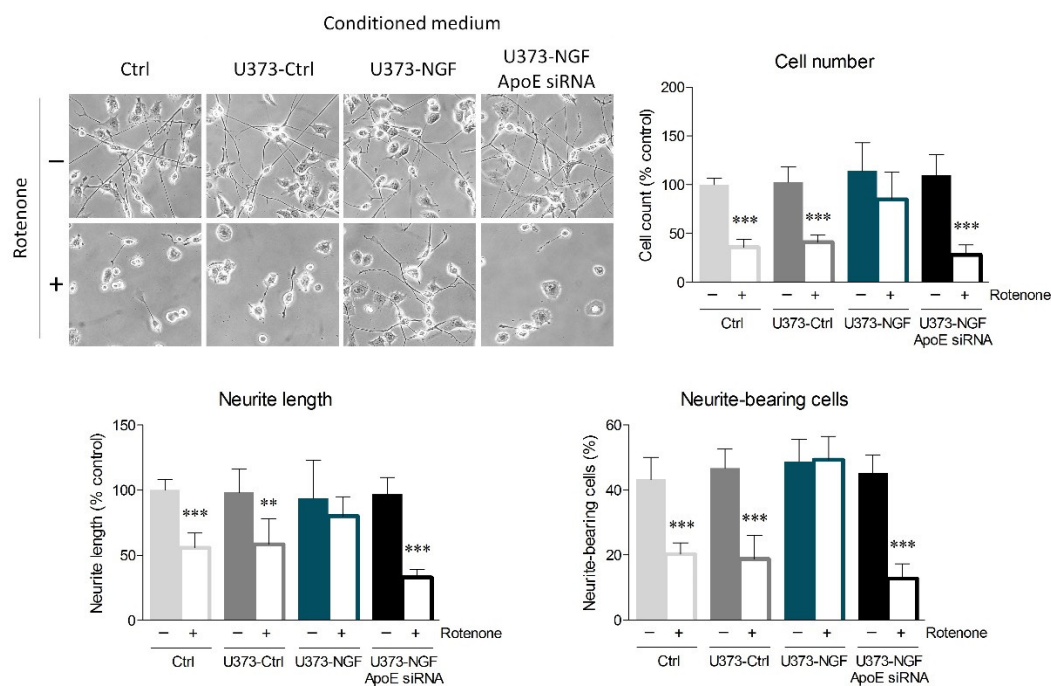


Figure 5. Effects of U373 conditioned medium on oxidative stress in neurons. Representative images in bright field and quantitative assessment of neuronal morphology of N1E-115. Cells were previously treated (+) or not (−) with rotenone (0.1 μ M) for 16 h, and then cultured in fresh DMEM (Ctrl), in conditioned medium derived from control U373 (U373-Ctrl), NGF-pre-treated U373 (U373-NGF), and NGF-pre-treated U373 silenced for apoE (U373-NGF ApoE siRNA) for 48 h. $n = 5$ different experiments. Data represent means $\pm$ SD. Statistical analysis was assessed by using one-way ANOVA, followed by Tukey's post hoc test. ** $p < 0.01$, *** $p < 0.001$.

To strengthen the results obtained from conditioned medium, we performed U373/N1E-115 co-cultures. To start, U373 cells were pre-treated or not with NGF for 48 h. Furthermore, differentiated N1E-115 cells were pre-treated or not with rotenone for 16 h. Subsequently, U373 medium was replaced with fresh medium containing 0.5% FBS, and coverslips seeded with N1E-115 were transferred onto the U373 layer to prepare co-cultures (Figure 6A). Oxidative damage elicited by rotenone treatment considerably affected cell viability, neurite-bearing cells, and neurite length of control N1E-115 and N1E-115 co-cultured with U373-Ctrl astrocytes (Figure 6B). Consistent with conditioned medium experiments, N1E-115 cells co-cultured with U373-NGF cells were protected from oxidative damage, whereas ApoE silencing in U373 abolished the neuroprotection sustained by NGF.

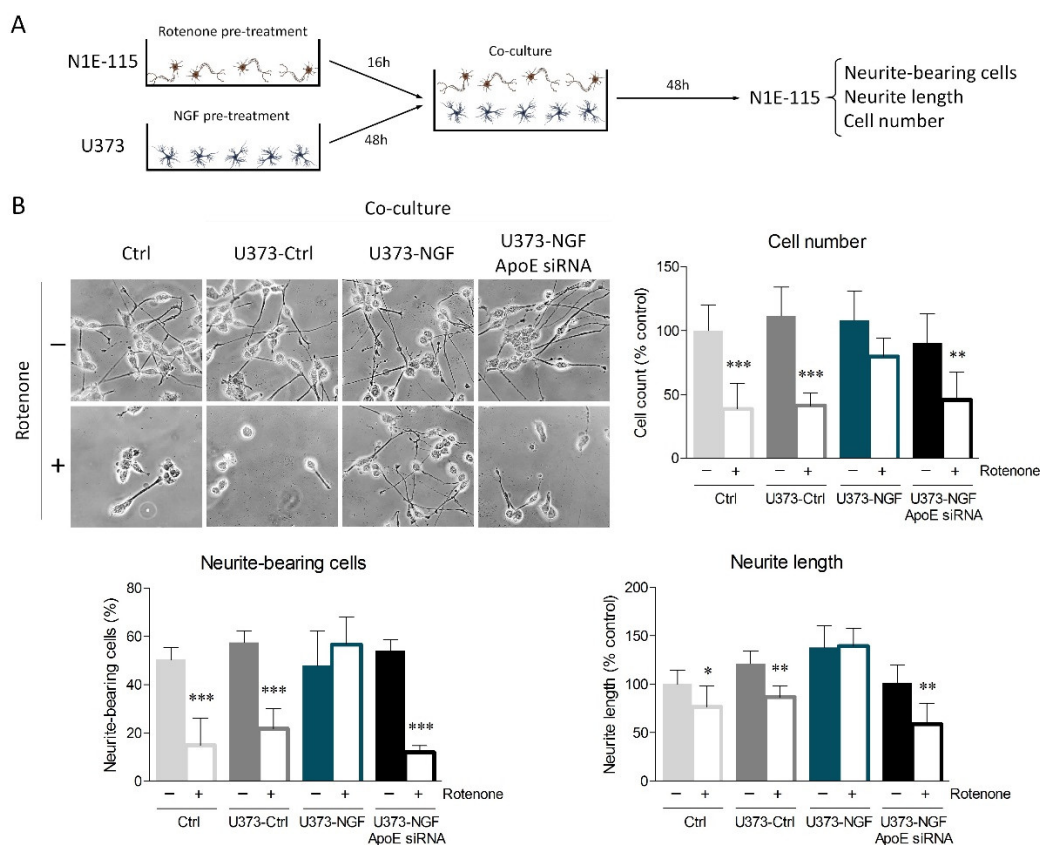


Figure 6. ApoE secretion by NGF-treated U373 cells provides neuroprotection from oxidative stress. (A) Representative scheme of astrocyte–neuron co-cultures set up as described in the Materials and Methods Section. Neurite-bearing cells, neurite length, and cell number were assessed 48 h after the establishment of co-culture. (B) Representative images in bright field and quantitative assessment of neuronal morphology of N1E-115 cells, previously treated (+) or not (–) with rotenone (0.1 μ M) for 16 h. N1E-115 were then kept in fresh DMEM (Ctrl), or co-cultured with control U373 (U373-Ctrl), NGF-pre-treated U373 (U373-NGF), and NGF-pre-treated U373 silenced for ApoE (U373-NGF ApoE siRNA) for 48 h. $n = 5$ different experiments. Data represent means $\pm$ SD. Statistical analysis was carried out by using one-way ANOVA, followed by Tukey’s post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. Discussion

Imbalances in neurotrophin signaling pathways, as well as alterations in brain cholesterol metabolism, are strongly associated with neurologic and neurodegenerative diseases, such as Rett syndrome, HD, Alzheimer’s disease (AD), and Parkinson’s disease (PD) [2,38,39]. In this context, fundamental research on the biological activity of neurotrophins is pivotal to better dissect the molecular mechanisms linking cholesterol metabolism and brain physiopathology. During the last few years, an interesting study reported a role for BDNF in the regulation of cholesterol metabolism in astrocytes [38]. Despite this notion, no information is available about the putative involvement of NGF in the regulation of astrocytic cholesterol. Therefore, in this work we focused on the prospective role exerted by NGF in the regulation of cholesterol metabolism, with particular reference to the influence of ApoE secretion on neuronal differentiation and survival. U373, the astrocytoma cell line used as an experimental model of human astrocytes, retains the potential to fully respond to NGF, as it expresses appreciable levels of both TrkA and p75NTR. The main results indicated that NGF upregulates the levels of the main proteins involved in cholesterol biosynthesis (HMGCR), intracellular trafficking (NPC1), and secretion (ABCA1). These events are accompanied by a concurrent increase of ApoE and cholesterol levels into the culture medium, suggesting that NGF increases cholesterol biosynthesis and extrusion from glial cells. It has been reported that NGF

induces ApoE protein expression by increasing its transcription [40]. However, we did not observe any significant change in intracellular ApoE expression. This apparent inconsistency can be explained since ApoE buildup cannot be appreciated because of its increased efflux from NGF-treated U373. With the attempt to better dissect the molecular mechanisms, we treated U373 with the LM11A-31 and found that the p75NTR modulator was only able to mimic the rise in HMGCR expression mediated by NGF. The involvement of p75NTR in the modulation of HMGCR levels is consistent with previous data obtained in other cell types, showing that this receptor controls the transcription of cholesterologenic enzymes [41]. However, LM11A-31 did not induce any changes either in the other proteins analyzed in this study, including ABCA1, or in cholesterol and ApoE levels into the culture medium, indicating that the selective modulation of p75NTR is not sufficient to promote cholesterol extrusion through ApoE-rich particles observed in NGF-treated cells. These results lead us to hypothesize that NGF governs cholesterol metabolism in glial cells through the involvement of both p75NTR and TrkA receptors. It is well known that neurotrophin binding to Trk receptors activates ERK1/2, thus regulating several pathways involved in cell differentiation, proliferation, and survival [2,38]. Interestingly, it has been observed that ERK1/2 activation also regulates ABCA1 and ApoE expression in different cell types [38,42,43]. These findings, together with the evidence that the BDNF/TrkB/ERK axis promotes ApoE extrusion and ABCA1 expression in glial cells [38], suggest that a signal transduction pathway involving the TrkA/ERK axis may explain a part of the effects exerted by NGF in our study.

While no changes were observed in neurite outgrowth, the employment of U373-conditioned medium and the establishment of co-culture experiments indicated that the increased ApoE secretion from glial cells mediated by NGF is determinant to prevent the detrimental outcomes induced by oxidative stress in neuronal cells. To induce cytotoxicity, we employed rotenone, a pesticide commonly used in preclinical research to induce mitochondria-derived reactive oxygen species (ROS) and subsequent apoptosis [44]. Our results highlight that NGF stimulates ApoE secretion by glial cells, which is necessary to guarantee the neuroprotective effects against rotenone administration in differentiated N1E-115 cells. The role of ApoE in the prevention of oxidative stress is well-documented. For instance, ApoE depletion in mouse models exacerbates oxidative injury in brain tissue [45,46]. Accordingly, administration of exogenous ApoE protects against irreversible oxidative damage from hydrogen peroxide treatment by counteracting secondary glutamate toxicity [18]. In addition, ApoE-containing lipoproteins are effective in attenuating apoptosis induced by withdrawal of trophic support in retinal ganglion neurons [47]. In our study, the employment of rotenone suggests that ApoE-mediated neuroprotection, promoted by NGF-stimulated glial cells, may be relevant in the context of neurodegenerative conditions such as PD. Notably, rotenone reproduces dopaminergic neuronal degeneration similar to that observed in PD, both in animal models and cell cultures, including N1E-115-derived neurons [48–50]. Coherently, our results support previous evidence showing that ApoE elicits neuroprotection upon the administration of 6-hydroxydopamine (6-OHDA), another neurotoxin capable of inducing a PD-like phenotype [51]. Collectively, our study demonstrates that NGF is a crucial modulator of cholesterol metabolism and ApoE extrusion from glial cells. Most importantly, the enhancement in ApoE secretion promoted by this neurotrophin is required to assure neuroprotection against rotenone cytotoxicity. BDNF is the most abundant neurotrophin in the adult brain. Despite this evidence, it has been shown that NGF may play a crucial role not only during development, but also in the adult central nervous system. For instance, NGF influences hippocampal activity and, as a consequence, spatial memory and learning [52]. Other findings suggest that NGF exhibits a neuroprotective effect on nigrostriatal dopaminergic neurons, and that this activity may set the basis for novel NGF-based therapeutic approaches for PD [53]. According to this notion, it has been shown that the production of NGF is deeply regulated in astrocytes during adulthood and exerts modulatory actions on neuroinflammation, brain injury, and neurodegeneration [54–56]. In this context, it would be interesting to evaluate, in future studies, whether these effects could be mediated, at least in part, by the modulation of glial

cholesterol promoted by NGF. The present work presents some limitations that should be overcome in the next experimental investigations; for instance, it would be important to better dissect the molecular mechanisms linking NGF treatment and cholesterol metabolism in glial cells. A broad understanding is useful to identify specific molecular targets amenable to pharmacological manipulation that could be employed in neurodegenerative contexts. Furthermore, although U373 and N1E-115 cells are widely used as manageable cell lines to respectively reproduce astrocytic and neuronal features [27–29,57], these findings should be further confirmed in primary cell cultures. Although further efforts should be made to strengthen the involvement of neurotrophin signaling in brain cholesterol homeostasis, this work demonstrates for the first time that NGF may indirectly exert neuroprotection by influencing glial cholesterol metabolism.

4. Material and Methods

4.1. Cell Culture

N1E-115 neuroblastoma cells and U373 cells were cultured at 5% CO₂ in DMEM at high glucose (Merck Life Science, Milan, Italy), with 10% (*v/v*) fetal bovine serum (FBS) (Merck Life Science, Milan, Italy), and added with penicillin/streptomycin solution.

To induce neuronal differentiation, N1E-115 cells were seeded at 30% confluency and were switched to DMEM with 0.5% FBS for 96 h.

Astrocyte-conditioned medium was prepared as previously reported [58]. Briefly, U373 were seeded at 70% confluency in 6-wells and maintained in DMEM with 10% FBS for 24 h. Cells were washed 3 times with PBS (Merck Life Science, Milan, Italy) and incubated with serum-free DMEM for 24 h. The medium was collected, centrifuged to remove cell debris, and immediately used for conditioned-medium experiments or kept at 80 °C for subsequent use. In all the experiments, N1E-115 cells were cultured in U373-conditioned medium diluted with fresh DMEM (dilution ratio 1:1), with a final concentration of 0.5% FBS.

Astrocyte–neuron co-cultures were set up according to the protocol described by Ioannou and colleagues, with modifications [59]. Paraffin spacers were pressed onto coverslips to assure adherence; we used spacers on neuronal coverslips to efficiently prevent mechanical damage when the cells were placed facing each other. Coverslips were then sterilized in ethanol and coated with poly-D-lysine (Merck Life Science, Milan, Italy). N1E-115 were seeded on the coated coverslips at the desired density and, once ready for the co-culture, sterile forceps were used to lift the coverslips with N1E-115 neurons and place them face-down into 6-wells seeded with U373 astrocytes.

Pre-treatments on N1E-115 cells were carried out by administering 0.1 µM of rotenone (Sigma-Aldrich, Milan, Italy, R8875) for 16 h. Control cells received DMSO (dilution 1:1000 in cell culture medium) as vehicle. U373 cells were treated with NGF (Alomone Labs, Jerusalem, Israel, N-245) at the dose of 100 ng/mL for 48 h in all the experiments.

4.2. Lysate Preparation and Western Blot Analysis

U373 cells were sonicated (duty cycle 20%, output 3) in sample buffer (0.125 M Tris-HCl containing 10% SDS, protease inhibitor cocktail, pH 6.8) for 30 s in order to obtain a total lysate, as previously described [60,61]. Laemmli buffer was added, and samples were denatured at 95 °C for 5 min. Protein extracts (twenty micrograms of proteins) were resolved on SDS-PAGE, and transfer onto nitrocellulose membrane was performed by using a trans-blot turbo transfer system (Biorad Laboratories, Milan, Italy), as previously reported [62]. Subsequently, the membrane was incubated at room temperature for 1 h with 5% no-fat dry milk in Tris-buffered saline (25 mM Tris-HCl, 138 mM NaCl, 27 mM KCl, 0.05% Tween-20, pH 6.8) and probed overnight at 4 °C with the following primary antibodies: anti-p75NTR (Santa Cruz Biotechnology, Dallas, TX, USA, sc-271708, dilution 1:500), anti-TrkA (Santa Cruz Biotechnology, Dallas, TX, USA, sc-118, dilution 1:500), anti-SREBP-1 (Santa Cruz Biotechnology, Dallas, TX, USA, sc-8984, dilution 1:1000), anti-SREBP-2 (Abcam, Cambridge, UK, ab30682, dilution 1:1000), anti-HMGCR (Abcam, Cambridge, UK, ab242315, dilution 1:1000), anti-LDLr (Abcam, Cambridge, UK, ab30532, dilution 1:500),

anti-NPC1 (Novus Biologicals, Centennial, CO, USA, NB400-148, dilution 1:3000), anti-ABCA1 (Santa Cruz Biotechnology, Dallas, TX, USA, sc-58219, dilution 1:400), anti-ApoE (Santa Cruz Biotechnology, Dallas, TX, USA, sc-53570, dilution 1:400), anti-vinculin (Sigma-Aldrich, Milan, Italy, V9264, dilution 1:10,000), and anti- β -actin (Santa Cruz Biotechnology, Dallas, TX, USA, sc-47778, dilution 1:10,000). Membranes were successively incubated for 1 h at room temperature with HRP-conjugated secondary anti-mouse or anti-rabbit antibodies (Bio-Rad Laboratories, Milan, Italy). Protein-bound antibodies were visualized by clarity ECL Western blotting (Bio-Rad Laboratories, Milan, Italy, #1705061), and chemiluminescence was registered through the ChemiDoc MP system (Bio-Rad Laboratories, Milan, Italy). Densitometric analysis derived from Western blots was then carried out by using ImageJ version 1.52t (National Institutes of Health, Bethesda, MD, USA) software for Windows. Vinculin or β -actin were used as housekeeping proteins, which served as internal controls for protein loading. Densitometric calculations were obtained as arbitrary units, derived from the ratio between the intensity of protein band and the respective housekeeping protein.

4.3. Oil Red O Staining

U373 cells were seeded on poly-L-lysine (Sigma-Aldrich, P6282-5MG)-coated coverslips in 6-well plates, and Oil red O staining was performed as previously reported [60]. Briefly, cells were fixed in paraformaldehyde (4% solution) for 10 min and gently rinsed three times with PBS. Fixed cells were incubated with 60% isopropanol for 5 min and then washed with distilled water. Subsequently, cells were probed with 1 mL of Oil Red O working solution (Sigma-Aldrich, O1391-250ML) for 15 min at room temperature by putting the 6-well plate onto an orbital rotator shaker. After the incubation with the staining solution, wells were washed three times with distilled water to eliminate the excess stain. Coverslips were finally mounted with Fluoroshield mounting medium (Sigma-Aldrich, F6182), and Oil red O autofluorescence was visualized via confocal microscopy (TCS SP8; Leica, Wetzlar, Germany). Images were captured using Leica TCS SP8 equipped with a 63 $\times$ magnification and Leica LAS X Software (Milan, Italy). Quantification of Oil red O staining was performed through ImageJ software for Windows and calculated as mean fluorescence intensity per cell area.

4.4. Filipin Staining

Filipin staining was carried out using Filipin complex (Sigma-Aldrich, F9765). Filipin stock solution (10 mg/mL in PBS) was always prepared immediately before use. Cells were fixed in paraformaldehyde (4% solution) for 10 min and washed with PBS. U373 were then stained with 1 mL Filipin working solution (0.05 mg/mL in PBS) for 1 h in the dark, at room temperature. Subsequently, cells were washed three times with PBS, and coverslips were mounted with Fluoroshield mounting medium and immediately analyzed through confocal microscopy using a UV filter set (340–380 nm excitation). Images were acquired at 40X magnification. Filipin quantification was calculated as mean fluorescence intensity per cell area by using ImageJ software for Windows.

4.5. Cholesterol Quantification

Cholesterol quantification was performed by using a colorimetric assay (Cholesterol Quantitation Kit, MAK043, Sigma-Aldrich, Milan, Italy) following the manufacturer's instructions.

4.6. ELISA

ApoE levels in culture medium were assessed by using Human Apolipoprotein E ELISA Kit (Abcam, Cambridge, UK, ab108813), according to the manufacturer's instructions.

4.7. Immunofluorescence

Immunofluorescence of U373 cells was executed as previously described [60]. U373 were fixed in paraformaldehyde (4% in PBS) and probed overnight with primary antibodies:

anti-p75NTR (Santa Cruz Biotechnology, Dallas, TX, USA, sc-271708, dilution 1:100), and anti-TrkA (Santa Cruz Biotechnology, Dallas, TX, USA, sc-118, dilution 1:100). Subsequently, cells were incubated for 1 h with goat anti-mouse secondary antibody Alexa Fluor 555 (ThermoFisher Scientific, Waltham, MA, USA, A28180) and with goat anti-rabbit secondary antibody Alexa Fluor 488 (ThermoFisher Scientific, Waltham, MA, USA, A27034). DAPI staining was performed to visualize nuclei, and coverslips were finally mounted with Fluoroshield mounting medium. Samples were analyzed via confocal microscopy, as described above.

4.8. ApoE Silencing

ApoE mRNA silencing was performed on U373 cells using ApoE siRNA (Santa Cruz Biotechnology, Dallas, TX, USA, sc-29708) according to the manufacturer's instructions. The transfection was set up in a 6-well tissue culture plate where 100,000 cells per well were seeded in DMEM antibiotic-free growth medium supplemented with 10% of FBS for 24 h. For each well, siRNA Transfection Medium (Santa Cruz Biotechnology, Dallas, TX, USA, sc-36868) was added to siRNA Transfection Reaction solution, prepared using siRNA duplex and siRNA Transfection Reagent (Santa Cruz Biotechnology, Dallas, TX, USA, sc-29528). Cells were then washed once with siRNA Transfection Medium and covered with the previously made transfection mixture. Subsequently, the culture plate was incubated for 7 h at 37 °C in a 5% CO₂ incubator. DMEM growth medium with 20% of FBS and antibiotics (2-fold the normal concentration) was added to the transfection mixture in each well with a 1:1 ratio. Cells were incubated for 18 h, and then the medium was discharged and replaced with fresh normal growth medium; 24 h after this last step, cells were ready to be used for further experiments. The efficiency of ApoE silencing was assessed by RT-qPCR in U373 cells and by ELISA in the cell supernatant; ApoE siRNA efficiently prevented ApoE induction by NGF if compared to scramble siRNA (ApoE mRNA: 83% decrease; ApoE ELISA: 78% decrease). Notably, ApoE expression upon siRNA silencing was lower than the basal expression levels observed in control U373 (ApoE mRNA: 72% decrease; ApoE ELISA: 58% decrease) (Figure S3A,B).

4.9. RNA Extraction and Real-Time PCR

mRNA analysis was performed as previously reported [22,63]. Briefly, total RNA from U373 cells was extracted with TRI Reagent (Merck Life Science, Milan, Italy) according to the manufacturer's instructions. DNase treatment (Ambion, Thermo Fisher Scientific, Milan, Italy) was performed, and then RNA was purified using an RNA clean up Kit (Zymo, Italy). RNA was successively reverse transcribed to cDNA through a High-Capacity cDNA Reverse Transcription Cell Kit (Applied Bio-System, Foster City, CA, USA) and used for qPCR analysis. Primers used in qRT-PCR for *apoE* were: forward 5'-GGGTCGCTTTTGGGATTACCTG-3' and reverse 5'-CAACTCCTTCATGGTCTCGTCC-3'. Primers used in qRT-PCR for *gapdh* (chosen as reference gene) were: forward 5'-GTCTCCTCTGACTTCAACAGCG-3' and reverse 5'-ACCACCCTGTTGCTGTAGCCAA-3'. The production of the correct amplicon was assessed by the evaluation of the melting curve. Each biological sample was run in triplicate with SYBR green IQ reagent (Bio-Rad Laboratories, Milan, Italy) and using the CFX Connect detection system (Bio-Rad Laboratories, Milan, Italy).

4.10. Quantitative Assessment of Neuronal Morphology

Neuronal morphology in N1E-115 cells was estimated by capturing images with an inverted microscope. Morphometric analysis was carried out through ImageJ software for Windows, and the number of total cells (expressed as percentage variation of the control), average neurite length (expressed as percentage of the control) and neurite-bearing cells were estimated. For neurite-bearing cells, the percentage was calculated by dividing the number of differentiated cells with the total number of cells per field. N1E-115 were defined differentiated when they had at least one neurite with length equal to or greater

than the diameter of the soma. Morphological evaluation was performed on at least five independent experiments, in which at least three images were analyzed.

4.11. Statistical Analysis

All the results presented in this study were expressed as mean $\pm$ SD (standard deviation). Normal distribution of the data was checked by using the Shapiro–Wilk test. When two experimental groups were compared, unpaired Student's *t* tests were applied. However, when three or more groups were compared, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was employed. Concerning the experiments with rotenone, where two variables were present, two-way ANOVA followed by a Bonferroni post test was used. Values of $p < 0.05$ were considered to be statistically different. Statistical analysis was carried out using GraphPad Prism 5 (GraphPad, La Jolla, CA, USA) for Windows.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms23094842/s1>.

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GENERAL CONCLUSIONS

Cholesterol is a lipid molecule essential for all mammalian cells, both during development and adulthood. Particularly, CNS exhibits the highest cholesterol content compared to other tissues. Most of the cholesterol present in the adult brain is localized in two compartments: the plasma membranes of neurons and astrocytes, where this lipid influences cell morphology, stabilizes membrane receptors, and regulates synaptic transmission; and the myelin sheaths, particularly important for neuronal physiology since the discontinuous isolation of axons is essential for the saltatory conduction of the action potential (*Björkhem & Meaney, 2004*).

Cholesterol is essential for the formation of dendrites and stimulates the differentiation of presynaptic terminals. Despite neurons are able to grow and survive in the absence of cholesterol, distinct phases of neuronal differentiation require the supply of this important sterol (*Goritz et al., 2005*). Furthermore, cholesterol is involved in the formation and stability of synapses and in neurotransmitter release (*Lecis and Segatto, 2014*).

Thus, alterations in cholesterol homeostasis are associated with numerous CNS disorders. These pathologies include neurodegenerative and neurodevelopmental diseases, including Alzheimer's disease, Parkinson's disease, Niemann-Pick disease type C, Huntington's disease, amyotrophic lateral sclerosis (ALS) and Rett syndrome (*Jin et al., 2019; Segatto et al., 2019*).

Disparate *in vitro* and *in vivo* experimental studies have demonstrated that epigenetic mechanisms may exert pivotal roles in regulating cholesterol biosynthesis, uptake and esterification (*Meaney, 2014*). However, the involvement of BET proteins in the regulation of cholesterol homeostasis is still unclear. Our studies contributed to identify a new mechanism of epigenetic regulation of cholesterol metabolism. Through a loss-of-function approach, we demonstrated that BET inhibition significantly modulates the main enzymes and proteins involved in cholesterol metabolism (*Tonini et al., 2020*). Particularly, we observed that this regulation is involved in neuronal differentiation, a process deeply dependent on cholesterol homeostasis (*Cartocci et al., 2016*). The data obtained in this PhD thesis highlight that BET inhibition promotes neuritogenesis and these changes in cellular morphology and function are accompanied by rearrangements in cholesterol metabolism. These results support the literature data according to which neurons repress cholesterol biosynthesis and fulfill the need for this sterol by increasing the activity of receptors involved in the extracellular uptake (*Pfriege, 2003*).

The impact of BET inhibition was also evaluated on adult neurogenesis. The formation of new neurons becomes pronounced in the two major stem cell niches following BET blockade in adult mouse brains. This evidence allows us to better understand the role of these epigenetic factors in neuronal differentiation, offering the rationale for the development of new pharmacological therapies aimed at contrast the various neuropathological conditions characterized by an altered neurogenesis.

Neurons depend on cholesterol production by other cell types (*Pfriege*r, 2003). Specifically, the transport of cholesterol to neurons is mediated by an important interplay with astrocytes, in which cholesterol biosynthesis is particularly intense (*Pfriege*r, 2002; *Pfriege*r and *Unger*er, 2011).

Even if this mechanism of horizontal transport is well described, the endogenous molecules involved in the regulation of cholesterol production in astrocytes and its transport to neurons are still unknown.

In this scenario, this thesis elucidated the mechanisms by which NGF influences cholesterol metabolism in glial cells. In particular, NGF upregulates the main proteins involved in cholesterol biosynthesis, intracellular transport and efflux and indirectly affects neuronal differentiation and survival through the enhanced ApoE secretion.

Taken together, the data collected in this PhD thesis expand the knowledge concerning the regulation of cholesterol metabolism in brain cells, and set the basis for the identification of innovative molecular targets useful for designing novel therapeutic approaches to cure or alleviate the symptoms of diseases affecting the CNS.

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LIST OF PEER-REVIEWED ARTICLES PUBLISHED DURING THE PhD COURSE

1. Tonini C, **Colardo M**, Colella B, Di Bartolomeo S, Berardinelli F, Caretti G, Pallottini V, Segatto M. *Inhibition of bromodomain and extraterminal domain (BET) proteins by JQ1 unravels a novel epigenetic modulation to control lipid homeostasis*. International Journal of Molecular Sciences. **2020**; 21. **IF=6.208**

2. Colella B, **Colardo M**, Iannone G, Contadini C, Saiz-Ladera C, Fuoco C, Barilà D, Velasco G, Segatto M, Di Bartolomeo S. *mTOR inhibition leads to Src-mediated EGFR internalisation and degradation in glioma cells*. Cancers. **2020**; 12: 2266. **IF=6.639**

3. Pallottini V, **Colardo M**, Tonini C, Martella N, Strimpakos G, Colella B, Tirazza P, Di Bartolomeo S, Segatto M. *ProNGF/p75NTR axis drives fiber type specification by inducing the fast-glycolytic phenotype in mouse skeletal muscle cells*. Cells. **2020**; 9: 2232. **IF=7.666**

4. **Colardo M**, Segatto M, Di Bartolomeo S. *Targeting RTK-PI3K-mTOR axis in gliomas: an update*. International Journal of Molecular Sciences. **2021**; 22: 4899. **IF=6.208**

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