ANTIPSYCHOTIC MEDIATED CHANGES IN CNTNAP3 GENE EXPRESSION IN SK-N-SH CELLS

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CERTIFICAN:

Que **Dña. Cristina Pazos del Olmo** ha realizado bajo nuestra dirección el presente trabajo Fin de Máster titulado: "ANTIPSYCHOTIC MEDIATED CHANGES IN CNTNAP3 GENE EXPRESSION IN SK-N-SH CELLS".

Consideramos que dicho trabajo se encuentra terminado y reúne los requisitos necesarios para su presentación como Trabajo Fin de Máster por la interesada, al objeto de poder optar al título de Máster Oficial en Iniciación a la Investigación en Salud Mental por la Universidad de Cantabria.

Santander, a 18 de septiembre de 2015.

Fdo.: Paula Suárez Pinilla

Fdo.: Fuencisla Pilar Cuéllar

Somewhere, something incredible
is waiting to be known.

(Carl Sagan)

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1. SUMMARY, KEYWORDS AND ACRONYMS

1.1. SUMMARY

Introduction. Schizophrenia is one of the most common and severe psychotic disorders of all. A growing number of researchers understand the need of genetic studies to unravel the pathophysiological mechanisms of mental disorders and their treatments. In this sense, a recent research reported changes in the expression of 17 genes after antipsychotic treatment. Among these genes, ADAMTS2, CD177, CNTNAP3, ENTPD2, RFX2, and UNC45B were overexpressed in patients with schizophrenia. The expression of these genes reverted to control values after 3 months of antipsychotic treatment. We have focused our study in the CNTNAP3 gene that is implicated in neuron-glia communication, and belongs to the NCP family of genes, which have been associated with schizophrenia. The aim of this study is to describe the concentration and time dependent changes in CNTNAP-3 gene expression in SK-N-SH cells treated with four antipsychotics during 24 hours.

Methods. SK-N-SH human neuroblastoma cell line was used in the study. HeLa cervical carcinoma cells were only used to compare the differential expression of the six genes. Different concentrations of haloperidol, clozapine, risperidone and aripiprazole were administered to test concentration and time-dependent CNTNAP3 expression changes. After these treatments, we isolated the RNA, and performed the Reverse Transcription and quantitative real-time PCR of the samples.

Results and conclusions. CNTNAP3 gene expression was increased in a concentration-dependent manner after treatment with the first generation antipsychotic haloperidol and the second generation antipsychotics clozapine and risperidone, while the third generation antipsychotic aripiprazole produced a reduction of its expression after 24 hours. Further experiments are needed to clarify the antipsychotic modulation of CNTNAP3 expression associated to this pathology, using more appropriate cell models.

1.2. KEYWORDS

Schizophrenia, SK-N-SH, CNTNAP3, qPCR, gene expression.

1.3. ACRONYMS

- **ADAMTS2**: A Disintegrin-Like And Metalloprotease (Reprolysin Type) with Thrombospondin Type 1 Motif, 2
- **CD177**: CD177 Molecule
- **CNTNAP3**: Contactin Associated Protein-Like 3
- **ENTPD2**: Ectonucleoside Triphosphate Diphosphohydrolase 2
- **RFX2**: Regulatory Factor X, 2
- **UNC45B**: Unc-45 Homolog B
- **DSM-V**: Diagnostic and Statistical Manual for Mental Disorders, 5th Edition
- **ICD-10**: International Classification of Diseases, 10th Revision
- **FGA**: First Generation Antipsychotics
- **SGA**: Second Generation Antipsychotics
- **TGA**: Third Generation Antipsychotics
- **D_2**: Dopamine2-receptor
- **$5HT_{1A}$** and **$5HT_{2A}$**: Serotonin1A and 2A receptors
- **DTNBP1**: Dystrobrevin Binding Protein 1
- **DISC1**: Disrupted In Schizophrenia 1
- **NGR1:** Neuregulin 1
- **COMT:** Catechol-O-methyltransferase
- **NCP**: Neuroxin-IV/CNTNAP/Paranodin family
- **EMEM:** Eagle´s Minimun Essential Medium
- **FBS:** Fetal Bovine Serum
- **P/S:** Penicillin/Streptomycin
- **DMEM:** Dulbecco´s Modified Eagle´s Medium
- **PBS:** Phosphate Buffered Saline
- **DSMO:** Dimethyl Sulfoxide

- **BLAST:** Basic Local Alignment Search Tool
- **NCBI:** National Center for Biotechnology Information
- **NuPack:** Nucleic acid Package
- **ACTB:** β-Actin
- **GAPDH:** Glyceraldehyde 3-Phosphate Dehydrogenase
- **qPCR**: Quantitative Polymerase Chain Reaction
- **Ct:** Threshold Cycle
- **mRNA**: Messenger Ribonucleic Acid
- **cDNA**: Complementary Deoxyribonucleic Acid
- **hiPSCs:** Human Induced Pluripotent Stem Cells

2. INTRODUCTION

2.1. PSYCHOTIC DISORDERS AND SCHIZOPHRENIA

Psychosis is a serious mental disorder characterized by loss of contact with reality. This disorder affects both sexes equally and the onset usually occurs in late adolescence or young adulthood[1]. There are different types of psychotic disorders based on operational diagnostic criteria and described in terms of signs and symptoms[2]. The last version of the Diagnostic and Statistical Manual for Mental Disorders DSM-V (American Psychiatric Association, 2013) and Chapter V (Mental and behavioral disorders) of the International Classification of Diseases ICD-10 (WHO, 1992) are the most used diagnostic systems today. Psychotic disorders are classified there based on the duration, dysfunction and type of delusions and hallucinations, in a wide range of disorders which include schizophrenia and its different clusters[2,3].

Schizophrenia is one of the most common and severe psychotic disorders of all. According to different studies, the lifetime prevalence of schizophrenia and any other psychotic disorders is above 3%[1,2]. Despite the great variety in courses and symptoms between patients, schizophrenia causes important lifetime disabilities and deterioration in functional capacity in every case[4].

The different clusters of schizophrenia disorders are characterized by disturbed thinking, perception and emotions[2], and by a variety of symptoms that can be classified in four main categories: *positive symptoms* – the so well-known delusions and hallucinations–; *negative symptoms* –such as lack of motivation or social withdrawal–; *cognitive symptoms* –alterations in neurocognition with attention or memory deficits- and *affective symptoms*, where affective dysregulation can lead to depressive or even bipolar symptomatology[5]. As we will see later, antipsychotic drugs work especially on positive symptoms by reducing or eliminating them, but are less effective with negative and cognitive symptoms[4].

2.2. PHARMACOLOGICAL TREATMENT: THE DOPAMINE HYPOTHESIS

The first medication used to treat schizophrenia disorders was discovered by serendipity in the early 1950s, leading to the *dopamine hypothesis* which considers schizophrenia a dopamine disorder: overactivity of the mesolimbic pathway –which explains the positive symptoms– and hypofunction of dopaminergic neurotransmission in the mesocortical pathway[6] –which explains the negative and cognitive symptoms– (Figure 1).

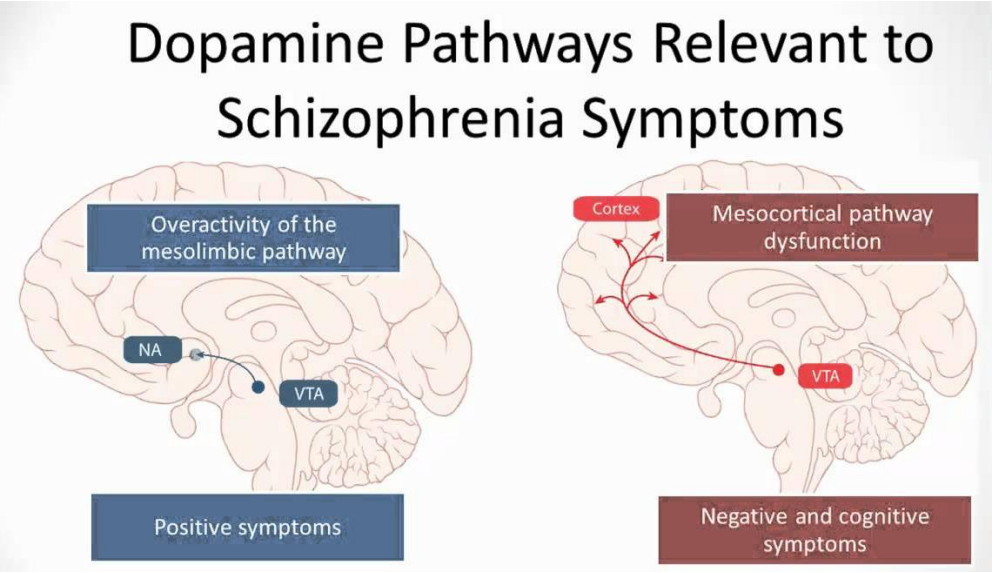

Figure 1. Dopamine pathways implicated in schizophrenia[6].

This hypothesis was developed as a result of the therapeutic effects of typical antipsychotics or first generation antipsychotics (FGA), which decrease the positive symptoms by blocking the dopamine D_2 receptors[4]. Nevertheless, **haloperidol** and other typical antipsychotics have been commonly replaced by the second generation antipsychotics or atypical antipsychotics (SGA), due to the many adverse side effects of FGA: extrapyramidal side effects, histaminic effects or anticholinergic effects, among others[7].

Atypical antipsychotics act both on dopamine D_2 and serotonin 5-HT_{2A} receptors, as antagonists. These drugs present fewer extrapyramidal side effects than FGA.

However, they show equal efficiency regarding positive symptoms and almost the same lack of effect on negative or cognitive symptoms than the typical antipsychotics[8]. **Clozapine** or **risperidone** are two examples of atypical antipsychotics.

It was only at the beginning of this century that a new type of antipsychotics was finally able to improve negative, cognitive and even mood symptoms. This type, known as the third generation antipsychotics (TGA), is also atypical as it acts on dopamine as well as serotonin. Undoubtedly, **aripiprazole** is the prototype and the most common antipsychotic of this group[9]. The main difference with the conventional atypical antipsychotics is that it works as a partial agonist –partial efficacy at the receptor compared to a full agonist- on both dopamine D_2 and serotonin $5HT_{1A}$ receptors, which makes it a dopamine-serotonin system stabilizer: it lowers dopaminergic neurotransmission in the mesolimbic pathway but it enhances dopaminergic activity in the mesocortical pathway, as well as on $5HT_{2A}$ receptors as an antagonist[6].

A comparison between the effect on dopamine and serotonin systems of the three types of antipsychotic drugs can be found in Figure 2.

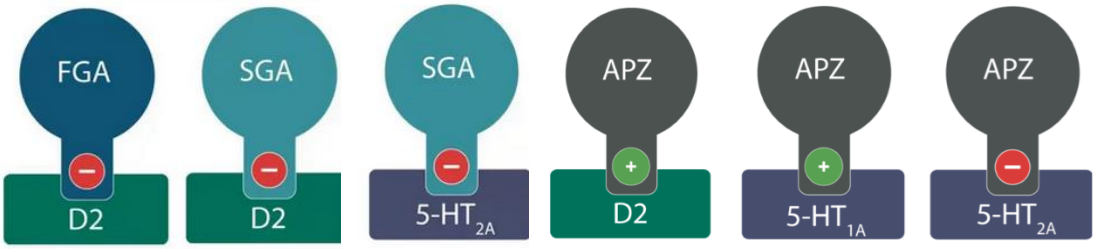

Figure 2. Effects on dopamine and serotonin systems of first (FGA, dark blue), second (SGA, light blue) and third (APZ, grey) generation antipsychotics. First generation drugs reduce dopaminergic neurotransmission in the four dopamine pathways (mesocortical, mesolimbic, nigrostriatal and tuberoinfundibular). Second generation reduce all four dopamine pathways by blocking D_2 as well, but the main difference with first generation antipsychotics is that they also work as $5HT_{2A}$ antagonists, which increases the nigrostriatal pathway activity and therefore reduces the risk of extrapyramidal side effects; moreover, it increases the dopamine release in prefrontal cortex, which could be responsible for the improvement of negative and cognitive symptoms. Finally, aripiprazole, the most common third generation antipsychotic, is a D_2 partial agonist acting over the mesocortical (increasing its activity) and the mesolimbic pathway (decreasing its activity). It also works on the serotonergic pathway as a $5HT_{1A}$ partial agonist and as a $5HT_{2A}$ antagonist[6].

These stabilizers show few neurological adverse effects and no side effects related to serum prolactin altered concentrations or weight gain.

A detailed description of the mechanism of action of the four highlighted antipsychotics is shown in Table 1.

Table 1. Mechanisms of action to different receptors of four antipsychotics (haloperidol, clozapine, risperidone and aripiprazole). Italics and bold text indicate primary targets of these antipsychotics[10].

	Haloperidol	**Clozapine**	**Risperidone**	**Aripiprazole**
H1 receptor	Antagonist	***Antagonist***	Antagonist	Antagonist
H4 receptor		Antagonist		
5HT1A receptor	Antagonist	Partial agonist	Antagonist	***Partial agonist***
5HT1B receptor		Full agonist	***Antagonist***	Full agonist
5HT1D receptor	Antagonist	Full agonist	***Antagonist***	Full agonist
5HT1E receptor		Full agonist	Antagonist	
5HT1F receptor		Full agonist	Antagonist	
5HT2A receptor	***Antagonist***	***Inverse agonist***	***Inverse agonist***	***Antagonist***
5HT2B receptor	Antagonist	Antagonist		
5HT2C receptor		Inverse agonist	Inverse agonist	Full agonist
5HT5A receptor		Antagonist		
5HT6 receptor		Inverse agonist	Antagonist	
5HT7 receptor	Antagonist	Inverse agonist	Inverse agonist	
α1A-adrenoceptor		Antagonist	Antagonist	
α1B-adrenoceptor		Antagonist	Antagonist	
α1D-adrenoceptor		Antagonist	Antagonist	
D1 receptor	Antagonist	Antagonist		
D2 receptor	***Antagonist***	Antagonist	***Antagonist***	***Partial agonist***
D3 receptor	***Antagonist***	Antagonist	Antagonist	
D4 receptor	***Antagonist***	Antagonist		
D5 receptor	Antagonist	Antagonist		

Despite the use of antipsychotics, it is known that more than a quarter of patients suffering from schizophrenia do not show any improvement with this medication. Additionally, the numerous adverse side effects of the medication and the little effectiveness on negative and cognitive symptoms –especially with first and second generation antipsychotics– reveal the many limitations of these drugs[7].

2.3. Schizophrenia as a Neurodevelopmental Disorder

After the dopamine hypothesis, there was a need to focus on cognitive symptoms, which led to the understanding of schizophrenia as a *glutamate disorder.* From this perspective, the cognitive symptoms of the disorder can be explained as a result of low activity of the NMDA glutamate receptors[7]. Nevertheless, just as in the case of dopamine, there is still no clear evidence that the abnormal neurotransmitter activity is the real –or, at least, the unique– cause of schizophrenia[4].

The attention in this century has changed from neuropharmacology to *genetics*, where multiple family and twin studies have demonstrated high heritability estimated at up to 80%[11]. At the moment, more than 40 candidate genes have been related directly to schizophrenia, but they still have not brought any light to the understanding of individual´s risk for this disorder. In addition to heritability, single nucleotide variations and rare structural variants studies show an effect on genes associated to brain development and may explain some of the risk for developing schizophrenia. However, most of these mutations are not specific to the disorder, which makes it difficult to believe in a simple genetic cause of schizophrenia[4].

All of these lead us to the understanding of the syndrome as a *neurodevelopmental disorder*, which not only does not exclude the other theories previously mentioned, but it groups all of them together. From this model, there is an abnormal brain development starting in the prenatal period and continuing until late adolescence, which includes a reduction of grey-matter volume and altered excitatory-inhibitory balance in the prefrontal cortex due to reduced myelination[4]. This abnormal

functioning is a result of genetic predisposition and early adverse events such as mid-gestational insults[12]. Thus, this explanation includes genes and genetic variants and mutations as a precursor to the disorder, always with the presence of adverse events, while it explains the altered functioning of neurotransmitters as a consequence of the abnormal brain development[4,12]. In other words, the combined effect of genes and environment.

2.4. Schizophrenia Gene Expression and Antipsychotics

As mentioned before, there are many studies that associate altered gene expression with schizophrenia. For instance, DISC1 gene variations have been associated to the disorder in many investigations and it has been recently suggested that these gene variations modulate the clinical severity of the disorder at the onset[13]. Other candidates include DTNBP1, NRG1 or COMT, among many others[14,15,16]. Furthermore, a growing number of researchers understand the need of genetic studies and the importance of genetic biomarkers in mental disorders[17,18].

Yet, there are not so many studies which can evidence a reversion to basal levels of these genes after the use of antipsychotic drugs. In this sense, a very recent research analyzing gene expression by next-generation sequencing was performed in blood samples of 22 schizophrenia patients before and 3 months after medication with atypical antipsychotics[19] –risperidone and aripiprazole among others–. The authors reported changes in the expression of 17 genes after antipsychotic treatment. Among these genes, six (ADAMTS2, CD177, CNTNAP3, ENTPD2, RFX2, and UNC45B) were overexpressed genes in patients with schizophrenia, as they are part of 200 genes that an earlier study highlighted as candidate genes associated to schizophrenia after they found significant differential expression in these genes in drug naïve schizophrenia patients compared to healthy matched controls[11]. These six genes are suggested to be implicated in the positive symptoms of the disorder, as they could be modulated by atypical antipsychotics[19]. These different levels of expression are shown in Figure 3.

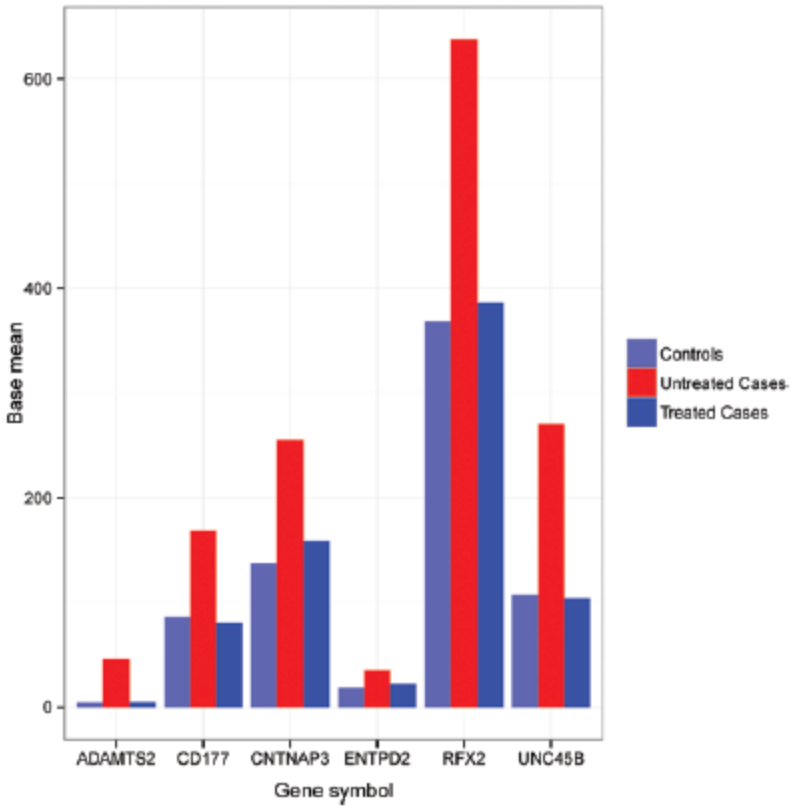

Figure 3. Expression levels in ADAMTS2, CE177, CNTNAP3, ENTPD2, RFX2 and UNC45B genes are increased in schizophrenia patients, and reverted by atypical antipsychotics[19].

2.5. NCP Family of Genes: CNTNAP3

CNTNAP3 is part of the NCP family (Neurexin-IV/Caspr/Paranodin), cell-recognition molecules which mediate neuron-glial interactions[20]. The identification of CNTNAP3 and CNTNAP4, also known as contactin associated proteins 3 and 4 (Caspr3 and Caspr4) respectively, is very recent. Both of them are expressed in nervous system. CNTNAP3 has been detected in myelinated axons in the corpus callous, the spinal cord, the cerebellum, temporal and frontal lobes, hippocampus (pyramidal cells of CA1, CA2 and CA3, and granular cells of the dentate gyrus), peripheral nerves and in oligodendrocytes. These two genes interact differentially with PDZ domain-containing proteins of the CASK/Veli/Mint complex, which are involved in cell recognition within the nervous system[20].

Caspr3 protein is formed by a large extracellular domain in the amino-terminus, a single membrane-spanning domain and a short cytoplasmic region in the carboxy-terminus. The extracellular region is composed of different domains: discoidin and fibrinogen-like domains, two EGF repeats and four laminin G domains (Figure 4). There are also two alternatively spliced forms: Caspr3TM which lacks the EGF and part of the fibrinogen-like domain, and Caspr3S which in addition lacks two laminin G domains and one EGF, together with the transmembrane domain (Figure 4). The Caspr3S form may act as a secreted isoform of Caspr3[20].

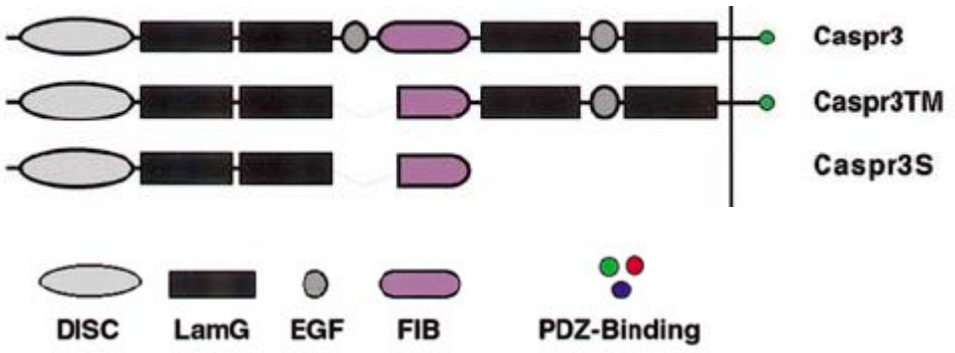

Figure 4. Alternative splicing generates two additional forms of Caspr3: Caspr3TM and Caspr3S, both missing exons 8–10, resulting in proteins that lack the first EGF domain and a small part of the fibrinogen-like region. Caspr3S lacks also exons 13–24 and may represent a secreted isoform of Caspr3. Discoidin-like domain (DISC); a region similar to fibrinogen (FIB); two EGF repeats; a short cytoplasmic domain that contains a carboxy-terminal binding site for PDZ domain[20].

The function of CNTNAP3 has not been fully described yet, although it seems to be associated to ion channel localization and function[20]. This gene has been suggested to be involved in some pathologies as Crohn´s Disease, as a recent study found that CNTNAP3 expression was upregulated in the intestinal tissue of these patients[21]. Additionally, it may be also involved in the etiology of bladder exstrophy[22.]

Little is known about this gene so far and there is not actual proved evidence of any connection between CNTNAP3 and schizophrenia, apart from the previously described study from Crespo-Facorro[19]. However, CNTNAP2 has been associated with

different neuropsychiatric disorders that include schizophrenia[23,24] and others like epilepsy[24], mental retardation[23] or autism[25,26]. Furthermore, CNTNAP4 has been found to contribute to GABA and dopamine synaptic transmissions[27], neurotransmitters that are implicated in schizophrenia, and to be expressed mainly in brain[28]. These opens the possibility of a connection between the CNTNAP family and schizophrenia.

2.6. The Importance of Cell Lines in Genetic Research

Nevertheless, working with blood samples has its limitations, as we cannot completely eliminate the interaction of other medication or even other genes that can also have effects in the expression levels of ADAMTS2, CD177, CNTNAP3, ENTPD2, RFX2, and UNC45B, and therefore avoid the great variability of gene expression profiles in human blood samples[29]. Furthermore, we can´t control the possible differences in patients´ responses to antipsychotic medication, due to variability of pharmacokinetics[30]. In addition, results obtained after chronic drug treatments could be due to compensatory changes instead of to a direct effect of the treatment[31].

In this sense, the use of cell lines has major advantages over blood samples and other methods, such as the degree of simplicity that they offer, isolating them from any unwanted interaction[32]. Genes can be studied separately and the direct effect of antipsychotics can be addressed easier. Moreover, cell lines are capable of indefinite growth, which gives the opportunity to replicate experiments as many times as needed. In conclusion, it simplifies the study of such a complex thing as mental disorders.

2.7. SK-N-SH Cell Line Characteristics

SK-N-SH is a neuroblastoma cell line with epithelial morphology that grows in adherent culture. When differentiating, these cells adopt a neuronal phenotype, which makes them optimal for investigating potential pathways involved in neuronal differentiation[33].

SK-N-SH was derived from a vanillylmandelic acid producing neuroblastoma, possibly representing a variant of adrenergic neuroblastomas. It shows low levels of tyrosine hydroxylase but high levels of dopamine ß-hydroxylase, and it occasionally has cytoplasmic catecholamine granules[34].

Additionally, these cells are known to have adenosinergic, acetyl cholinergic, glutamatergic and dopaminergic activity[35]. SK-N-SH cells also present serotonergic receptors as 5-HT_{1A}[36] and 5-HT_{2A}[37]. These last activities make them interesting to study schizophrenia pathways, taking into account the dopamine and serotonin mechanisms of action of antipsychotics.

3. Hypothesis and Objectives

Our hypothesis is that CNTNAP3 gene expression is either directly modulated by antipsychotic drugs, supporting its role in schizophrenia; or a consequence of indirect processes secondary to the disease. Gene expression decreases due to the effect of atypical antipsychotics.

The aim of this study CNTNAP3 gene expression in SK-N-SH cell line (neuronal line) treated with different antipsychotics (first, second and third generation antipsychotics) during a 24 hour period in order to confirm or refute our hypothesis. This main goal can be divided in different specific objectives:

- To evaluate ADAMTS2, CD177, CNTNAP3, ENTPD2, RFX2, and UNC45B basal expression levels in a neuronal and in a non-neuronal cell line.
- To check the specificity of the CNTNAP3 qPCR primers.
- To evaluate the CNTNAP3 gene expression using different concentrations of the antipsychotics haloperidol, clozapine, aripiprazole and risperidone after 24 hours of incubation: **concentration-dependent expression**.
- Evaluate changes in CNTNAP3 gene expression at different time points of the incubation with the antipsychotics haloperidol, clozapine, aripiprazole and risperidone: **time-dependent expression**.

4. Material and Methods

4.1. Chemicals, Reagents and Drugs

The base medium for SK-N-SH cell lines is Eagle´s Minimun Essential Medium (EMEM) (purchased from American Type Cell Culture, VA, USA). To make the complete growth medium, the following components were added: 10% fetal bovine serum (FBS), 2% L-glutamine and 2% penicillin/streptomycin (P/S). These reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The base medium for HeLa cell lines is Dulbecco´s modified Eagle´s medium (DMEM). To make the complete growth medium, the following components were added: 10% fetal bovine serum, 2% L-glutamine and 2% penicillin/streptomycin. These reagents, including DMEM, where purchased Sigma-Aldrich (St. Louis, MO, USA).

Phosphate buffered saline (PBS), trypsin, TriPure™ Isolation Reagent and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). SYBR Green PCR Master Mix was purchased from Thermo Fisher Scientific (MA, USA). Primers were obtained from Sigma-Aldrich (St. Louis, MO, USA).

The antipsychotics haloperidol, clozapine aripiprazole and risperidone were ordered to Tocris Bioscience (Bristol, UK).

4.2. Cell Culture

SK-N-SH human neuroblastoma cells (Figure 5) were cultured in EMEM medium supplemented with 10% FBS, 2% L-glutamine and 2% P/S. Cells were seeded on flasks or standard microplate wells and were grown in a humidified incubator with 5% CO_2 at 37ºC. The culture medium was changed every 1-2 days.

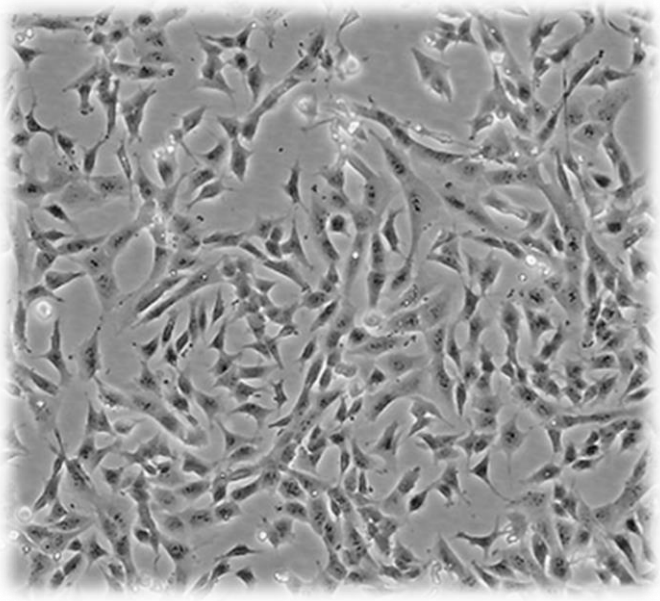

Figure 5. SK-N-SH human neuroblastoma cells view using phase contrast microscopy ×100.

HeLa cervical carcinoma cell lines were cultured in DMEM medium supplemented with 10% FBS, 2% L-glutamine and 2% P/S. Cells were seeded on flasks or standard microplate wells and were grown in a humidified incubator with 5% CO_2 at 37ºC. The culture medium was changed every 2-3 days.

Both cell lines were subcultured when they were approximately 80% confluent using Trypsin to detach cells from the plate surface, and seeding in microplate wells.

HeLa cell line was used only to compare the differential expression of the six genes studied in a non-neuronal cell line (HeLa), with a neuronal cell line (SK-N-SH).

4.3. Selection of Antipsychotics´ Concentrations

Antipsychotics´ concentrations were selected after an exhaustive search in the literature to establish the appropriate antipsychotics treatment concentrations to produce an effect on SK-N-SH cells without damaging cell culture[38,39,40,41,42,43,44,45,46,47]. Concentrations of haloperidol were considerably lower, as it produces toxicity at higher concentrations[38].

We carried out two main parts in the study, first one consisting in a concentration-dependent expression, where cells were exposed to different concentrations of antipsychotics for 24 hours. Second part consisted in a time-dependent expression, where the cell line was treated with a single concentration of each antipsychotic at different time points. More details of these concentrations and time points can be found in Table 2.

Table 2. Antipsychotics concentrations used in the different studies.

Antipsychotic	Concentration-dependent	Time-dependent (1, 2, 5, 8 and 24 hours)
Haloperidol	0.1, 0.25, 0.5 and 1 µM	0.5 µM
Clozapine	3.75, 7.5, 15 and 30 µM	15 µM
Risperidone	1, 2.5, 5 and 10 µM	10 µM
Aripiprazole	5, 10, 20 and 40 µM	40 µM

4.4. Treatment with Antipsychotics

Antipsychotics were dissolved with alcohol (haloperidol) and DMSO (clozapine, aripiprazole and risperidone) to an initial concentration of 10mM. These stock solutions were diluted in order to obtain 100 fold concentrated dilutions of the antipsychotics final concentration in the experiments. Cell cultures were treated 24 hours after cell plating.

For the concentration-expression part of the study, a single experiment was performed (n=1). For the second part, the temporal curve, the experiments were performed in triplicate (n=3).

In each experiment two basal conditions were included: a basal condition with the culture medium alone, and a second basal condition including ethanol or DMSO (depending on the antipsychotic used), to a final concentration similar to that used for the drugs conditions.

4.5. RNA Extraction

Isolation of total RNA was performed using TriPure™ Isolation Reagent (Sigma), following the manufacturer's instructions. The concentration and purity of the obtained RNA was quantified using a NanoDrop Spectrophotometer (Thermo Scientific, DE, USA).

4.6. Reverse Transcription (RT)

cDNA was obtained from the RNA by the Reverse Transcription reaction using a High Capacity cDNA Reverse Transcription kit (Applied BiosystemsTM, California, USA). Reverse Transcription reagent composition is indicated in Table 3A, and protocol steps are indicated in Table 3B. An Applied Biosystems 2720 Thermal Cycler was used to perform this reaction.

Table 3. RT reaction protocol indicating quantities (A) and reaction steps (B).

A	Quantities
10x RT Buffer	**2.0 µl**
25x dNTP Mix (100 mM)	**0.8 µl**
10x RT Random Primers	**2.0 µl**
MultiSribe^{TN} RT	**1.0 µl**
Nuclease-free H_2o	**4.2 µl**
RNA (30 ng / µl)	**10 µl**
TOTAL	**20 µl**

B	Temperature	Time
Cycle 1	25ºC	10 min
Cycle 2	37ºC	120 min
Cycle 3	85ºC	5 min
Cycle 4	4ºC	∞

4.7. Primers Design and Primers Sequencing

The NCBI Reference Sequence number for the six genes studied was obtained from the NCBI Gene Database (http://www.ncbi.nlm.nih.gov/gene/). For primer design we used the Primer BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/; Basic Local Alignment Search Tool, Bethesda, MD, USA). Finally, we performed a

thermodynamic Analysis of Interacting Nucleic Acid Strands using NuPack (Nucleic acid Package; http://www.nupack.org/), with the primers obtained for the six genes to check possible secondary structures.

The primers for the six genes studied are detailed in Table 4. Figure 6 represents the CNTNAP3 primers location within the gene that comprises exons 20 and 21.

Table 4. Six genes primers sequence.

Gene	NCBI Reference Sequence	Primer pair	Primer sequence
ADAMTS2	NM_014244.4	Forward	5´ CCTGACATCCTCAAACGGGA 3´
		Reverse	5´ GTGTGGGTTGTCACACTGGC 3´
CD177	NM_020406.3	Forward	5´ CATGTGTGGAAGGTGTCCGA 3´
		Reverse	5´ TGAGCATCAACGTGTCCTGG 3´
ENTPD2	NM_001246.3	Forward	5´ GCCTTCTCTGCCTTCTTCTACA 3´
		Reverse	5´ GTTGCAGACATTCACTGCGG 3´
RFX2	NM_000635.3	Forward	5´ CAGCAGAGCTCCCTGGAC 3´
		Reverse	5´ TTCAGCAAGAACTGCCGGG 3´
UNC45B	NM_001033576.1	Forward	5´ GGACTCTGTAAGCTCGGCTC 3´
		Reverse	5´ CCACTTGCGACACTGTTTGG 3´
CNTNAP3*	NM_033655.3	Forward	5’ TTAACCTCTACCATGACCAC 3’
		Reverse	5’ AGGTACAAGCTAGATAGAC 3’

* CNTNAP3 primers were predesigned by Sigma-Aldrich (KiCqStart™ Primers).

Figure 6. CNTNAP3 primers location within the gene.

4.8. Housekeeping Genes

Two housekeeping genes were used to normalize CNTNAP3 gene expression: β-actin (ACTB), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). These constitutive genes present stable and homogenous expression in all cells of the organism[48] and, therefore, are optimal for this study. Housekeeping primers sequences are shown in Table 5.

Table 5. Housekeeping primers sequence.

Housekeeping gene	Primer pair	Primer sequence
ACTB	Forward	5´TAAAAACTGGAACGGTGAA3´
	Reverse	5´ACAACGCATCTCATATTTGG3´
GAPDH	Forward	5´ACAGTTGCCATGTAGACC3´
	Reverse	5´TTTTTGGTTGAGCACAGG3´

4.9. Quantitative PCR (qPCR)

Quantitative polymerase chain reaction (qPCR) or real-time polymerase chain reaction was performed on an Applied Biosystems 7500 Fast Real-Time PCR System using SYBR Green Master Mix. The qPCR reaction composition is indicated in Table 6A, and the protocol steps including the cycles are shown in Table 6B.

Table 6. qPCR reaction composition (A) and protocol cycles (B).

A	Quantities
$CNTNAP_3$-F 10 µM	**0.6 µl**
$CNTNAP_3$-R 10 µM	**0.6 µl**
SYBR Green Mix	**10 µl**
cDNA	**2 µl**
Nuclease-free H_2o	**6.8 µl**
TOTAL	**20 µl**

B	Temperature	Time
Cycle 1	95ºC	5 min
Cycle 2 (x40)	95ºC	10 sec
	60ºC	10 sec
	72ºC	10 sec
Cycle 3	4ºC	∞

Melting curves were evaluated for the different genes studied in order to check the specificity of the primers.

4.10. qPCR Primer Validation: Reaction Efficiency

In order to determine the efficiency of CNTNAP3 primers a standard curve was performed using different cDNA concentrations. The cDNA concentrations used for the experiment were 30, 10, 3, 1, 0.3, 0.1 and 0.03 ng/µl of cDNA. The efficiency of the qPCR assay should be 90–105%.

4.11. Data Analysis

The expression levels for each gene were normalized with respect to GAPDH and ACTB. The relative change in the mRNA levels of the CNTNPA3 gene between the antipsychotic-treated condition and the control conditions was determined by the equation[49]:

$$\text{Fold change} = 2^{-\Delta\Delta Ct}$$

$$\Delta\Delta Ct = (Ct\ CNTNAP3 - Ct\ HK)\ treated - (Ct\ CNTNAP3 - Ct\ HK)control$$

In addition, a one-way ANOVA followed by a Newman-Keuls post-hoc test was used to compare among the different time points.

5. RESULTS

5.1. ANALYSIS OF THE SIX GENES EXPRESSION PATTERN IN HELA AND SK-N-SH CELL LINES

Primers for the six genes studied were used to check basal gene expression in untreated HeLa and SK-N-SH cell lines. The expression order in HeLa cell line was: CD177 > RFX2 > ADAMTS2 > UNC45B > ENTPD2 > CNTNAP3; CNTNAP3 gene expression was almost undetectable in this cell line (Table 7). In SK-N-SH cells, the expression order was: CNTNAP3 > RFX2 ≥ UNC45B > ADAMTS2 > ENTPD2; CD177 expression was not detected in this cell line (Table 7). From the six genes studied, CNTNAP3 showed the highest expression difference between SK-N-SH and HeLa cell lines.

Table 7. qPCR Ct values of the genes studied in HeLa and SK-N-SH cell lines.

	HELA			SK-N-SH		
	Mean Ct		S.E.M.	Mean Ct		S.E.M.
ADAMTS2	32,064			33,852	±	
CD177	29,946	±	0,042	N.D.		
ENTPD2	35,122	±	0,413	37,061	±	
RFX2	31,384	±	0,075	31,010	±	0,316
UNC45B	34,119	±	0,431	31,419	±	0,104
CNTNAP3	38,334	±	0,003	26,688	±	0,392

N.D.: non-detectable.

5.2. CNTNAP3 PRIMERS OPTIMIZATION

To examine the efficiency of CNTNAP3 gene primers, cDNA serial dilutions were carried out (1, 1:3, 1:10, 1:30, 1:100, 1:300 and 1:1000). The relation between the threshold cycle (Ct) and the log (cDNA) was linear, with a slope value of -3.322 (r^2=0.98),

which indicates an efficiency of 100% (using the formula: Efficiency = $10^{(-1/slope)}$-1). Figure 7 shows the graph of this optimization.

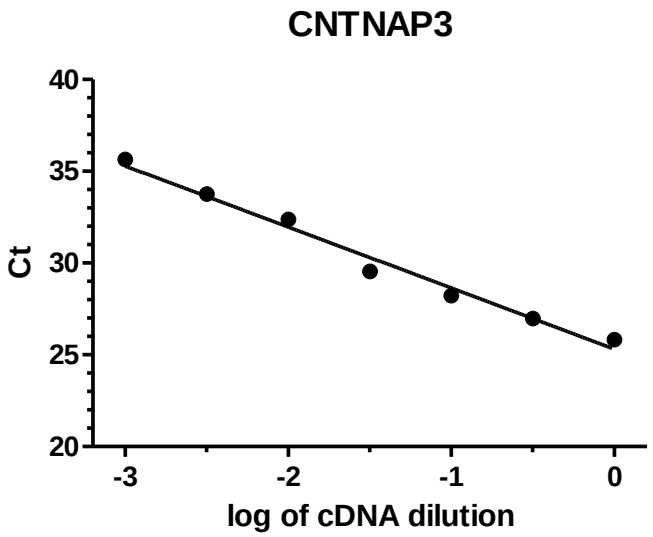

Figure 7. Standard curve to determine primers specificity.

5.3. Effects of Antipsychotic Drugs on CNTNAP3 Gene Expression on SK-N-SH Cell Line: Concentration-dependent Expression

A first part of the study consisted in testing different concentrations of four antipsychotics and its effects in CNTNAP3 expression after 24 hours. Depending on the antipsychotic used in the respective treatments, the results were divided in first, second and third generation antipsychotics.

5.3.1. First Generation Antipsychotic (Haloperidol)

SK-N-SH cells were treated for 24 hours with 0.1, 0.25, 0.5 and 1 µM concentrations of haloperidol. CNTNAP3 gene expression was set to 0 as the basal level in the control condition. As we can see in Figure 8, there was a small increase (9% over basal values) of CNTNAP3 expression with 0.1 µM haloperidol, and a higher increase with 0.5 and 1 µM conditions (34% and 27% respectively over basal values).

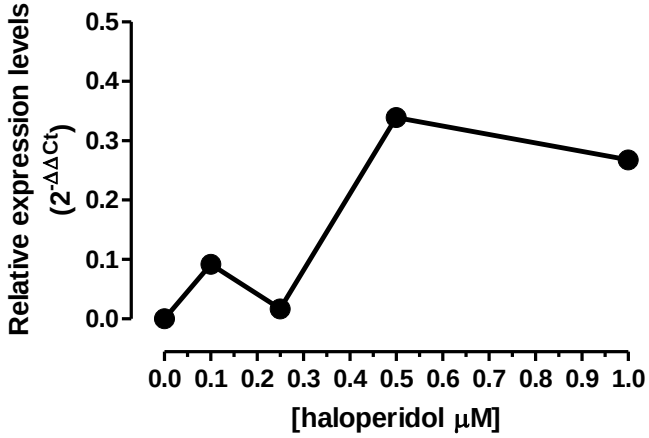

Figure 8. CNTNAP3 gene expression in SK-N-SH cells after 24 hours of treatment with different concentrations of haloperidol.

5.3.2. Second Generation Antipsychotics (Clozapine and Risperidone)

SK-N-SH cells were treated for 24 hours with 3.75, 7.5, 15 and 30 μM concentrations of clozapine, and 1, 2.5, 5 and 10 μM concentrations of risperidone. CNTNAP3 gene expression was set to 0 as the basal level in the control condition. As we can see in Figure 9A, there was an initial small decrease of CNTNAP3 with 3.75 μM dose of clozapine (-7% *vs* basal values), but a subsequent slight rise with 7.5 μM concentration (5% *vs* basal values), which continues with 0.5 and 10 μM concentrations of clozapine (29% and 27% respectively *vs* basal values).

With regard to risperidone, all concentrations produced an increase in CNTNAP3 gene expression: 1 μM with 51%, 2.5 μM with 39%, 5 μM with 64%, and 10 μM with 46% *vs* basal values (Figure 9B).

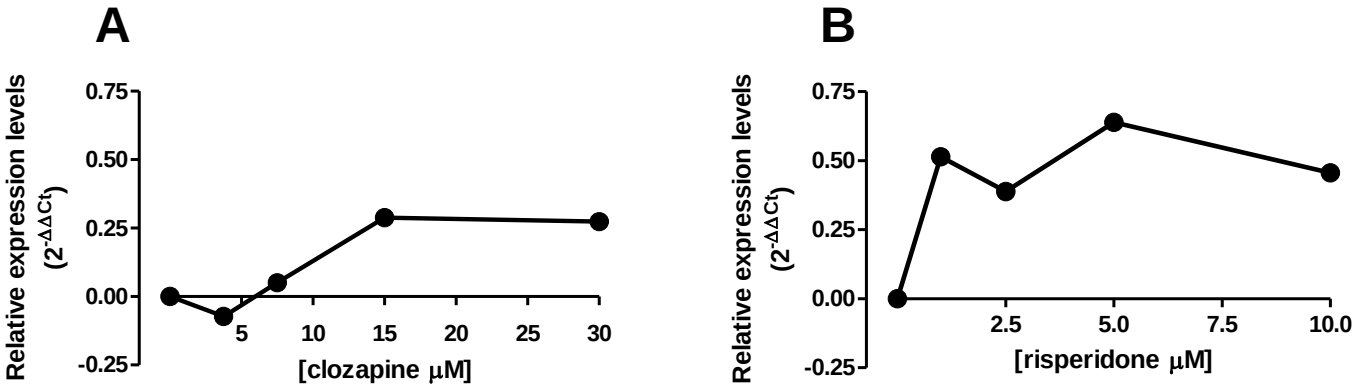

Figure 9. CNTNAP3 gene expression in SK-N-SH cells after 24 hours of treatment with different concentrations of clozapine (A) and risperidone (B).

5.3.3. Third Generation Antipsychotic (Aripiprazole)

SK-N-SH cells were treated for 24 hours with 5, 10, 20 and 40 µM concentrations of aripiprazole. CNTNAP3 gene expression was set to 0 as the basal level. As we can see in Figure 10, there was a small increase of CNTNAP3 with 5 µM dose of aripiprazole (11% *vs* basal values), and a subsequent decrease with higher concentrations (10 µM with -10%, 20 µM with -46%, and 40 µM with -26% *vs* basal values).

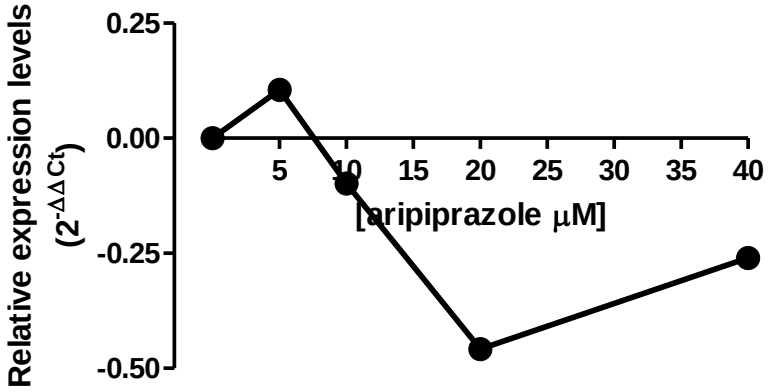

Figure 10. CNTNAP3 gene expression in SK-N-SH cells after 24 hours of treatment with different concentrations of aripiprazole.

5.4. Effects of Antipsychotic Drugs on CNTNAP3 Gene Expression on SK-N-SH Cell Line: Time-dependent Expression

A second part of the study was the time course of CNTNAP3 expression in SK-N-SH cells after antipsychotic treatment during different time points within 24 hours (1, 2, 5, 8 and 24 hours). We have also divided this in first, second and third generation antipsychotics. The antipsychotics concentration used in these experiments was obtained from the above described results, selecting a concentration in which a higher expression level change was obtained.

5.4.1. First Generation Antipsychotic (Haloperidol)

SK-N-SH cells were treated to study the time-dependent effect of 0.5 µM of haloperidol. CNTNAP3 gene expression was set to 0 for the basal level. As we can see in Figure 11, there was a small increase of CNTAP3 after one hour (5% *vs* basal values), 5 hours (8% *vs* basal values), 8 hours (21% *vs* basal values) and 24 hours (50% *vs* basal values).

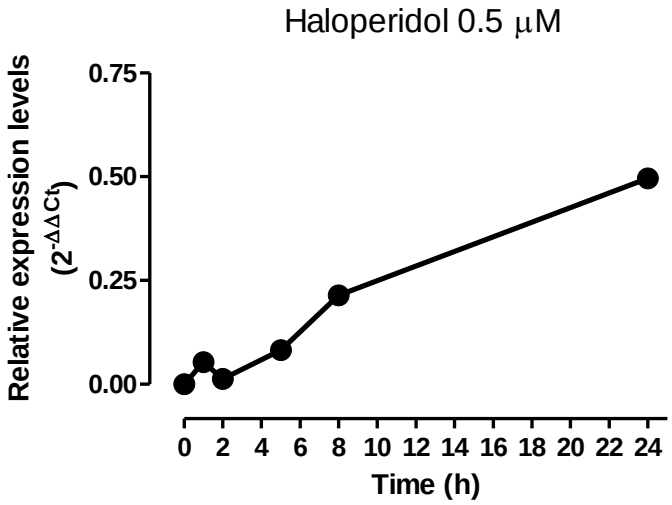

Figure 11. CNTNAP3 gene expression in SK-N-SH cells during a 24 h period of treatment with 0.5 µM haloperidol, evaluating different time points.

5.4.2. Second Generation Antipsychotics (Clozapine and Risperidone)

SK-N-SH cells were treated with 15 µM clozapine and 10 µM risperidone, and samples were collected at different time points during 24 hours. CNTNAP3 gene expression was set to 0 as the basal level. Figure 12 shows the results.

In the case of clozapine, the expression of CNTNAP3 gene reached a maximal expression after the first hour (16±12% *vs* basal value), and decreased until 8 hours

incubation time (2 hours: 11±7%, 5 hours: 9±13% and 8 hours: -6±14% *vs* basal value). The expression level until 24 h remained close to basal values (-3±17% vs basal value).

Regarding risperidone, the time-dependent changes follow a similar pattern to clozapine, although gene expression values are in general of a higher magnitude. The expression of CNTNAP3 gene increased after the first hour (33±10% *vs* basal value), and levels went down until 8 hours incubation (2 hours: 27±9%, 5 hours: 18±20% and 8 hours: -3±12% *vs* basal value). After 24 hours, the CNTNAP3 gene expression is increased to 22±13% *vs* basal value.

No significative changes were observed comparing the expression levels from the studied time points for each antipsychotic using a one-way ANOVA analysis.

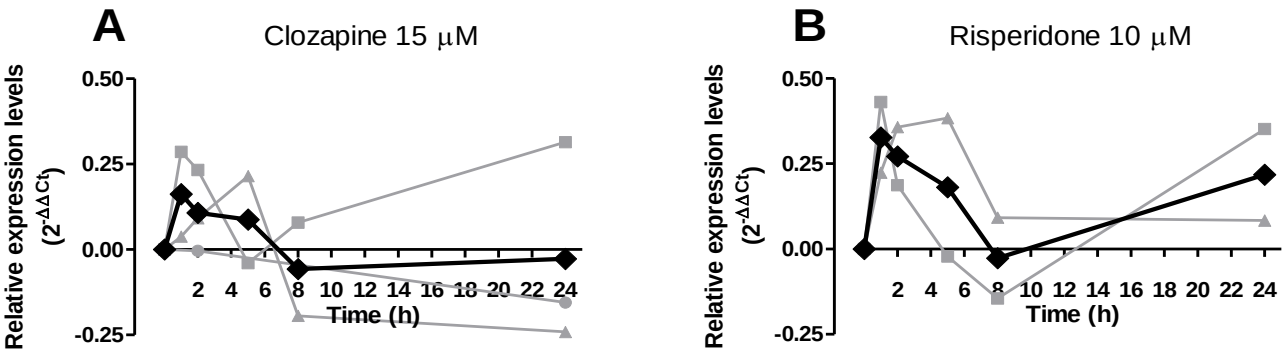

Figure 12. Time dependent CNTNAP3 gene expression in SK-N-SH cells in a 24 hours period of treatment with 15 μM clozapine (A), and 10 μM risperidone (B) concentrations. Results for individual experiments are shown in grey, and mean values in black.

5.4.3. Third Generation Antipsychotic (Aripiprazole)

SK-N-SH cells were treated with 40 μM aripiprazole during different time points within 24 hours. Once more, CNTNAP3 gene expression was set to 0 as the basal level.

As shown in Figure 13, expression levels of CNTNAP3 gene decreased over basal values at all time-points (1 hour: -18±6%, 2 hours: -33±10%, 5 hours: -30±17%, and 24

hours: -26±6% *vs* basal value), with an increase in CNTNAP3 expression close to basal values after 8 hours (-8±15% *vs* basal values).

No significative changes were observed comparing the CNTNAP3 expression levels from the different time points of the aripiprazole treatment using one-way ANOVA analysis.

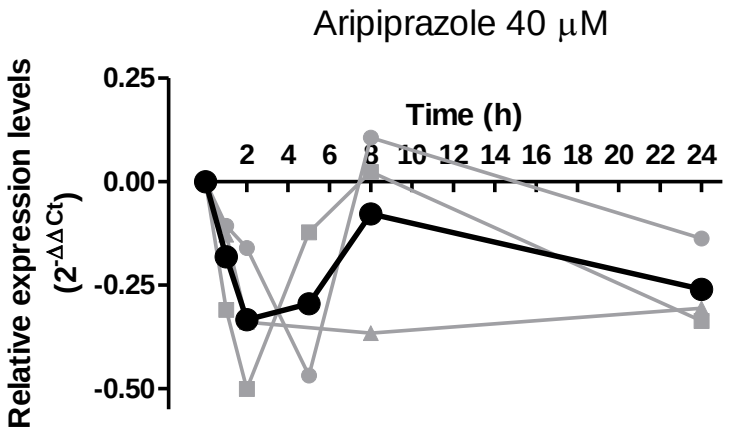

Figure 13. CNTNAP3 gene expression in SK-N-SH cells after different time points (up to 24 hours) of treatment with 40 μM aripiprazole concentrations. Results for individual experiments are shown in grey, and mean values in black.

6. DISCUSSION

In this study we have evaluated the direct and early changes in the expression of one of the six genes whose modulation has been recently associated to antipsychotic treatment, the CNTNAP3 gene[19]. The aim of this work was to study the early expression changes of this gene associated to the antipsychotic treatment, as the CNTNAP3 gene changes previously reported[19] could be a direct or an indirect consequence of antipsychotic treatment.

6.1. BASAL GENE EXPRESSION

The initial screening of the expression of genes ADAMTS2, CD177, ENTPD2, RFX2, UNC45B and CNTNAP3 in HeLa cell line –non-neuronal–, and in SK-N-SH cell line –neuronal–, let us determine cell-type specific differences in the expression of these genes[50]. The similar expression levels of ADAMTS2, ANTPD2, RFX2 and UNC45B genes in both cell strains imply a lack of cellular selectivity. In contrast, CD177 gene expression was not detected in the neuronal cell line, which suggests that changes reported in schizophrenia patients could be a consequence of somatic changes and not directly related to the mental disorder, as genes linked to cardiovascular risk[51].

The CNTNAP3 gene showed a very low expression in HeLa cell line and the highest expression in SK-N-SH cell line. The higher CNTNAP3 expression in a neuronal cell line justifies the use of this gene in this study. CNTNAP3 is implicated in the neuron-glia recognition and communication[20]. This interaction may have an outstanding role, since it has been described the neuron-glia impairment in animal models of this pathology that resemble positive, negative and cognitive symptoms[52]. Moreover, other members of the family as the CNTNAP2 gene have been also associated to schizophrenia[23,24].

In this study we have studied the CNTNAP3 genes expression using a set of primers specific for a sequence ranging exons 20-21, that is able to detect the complete form of the protein, and a truncated form known as CNTNAP3TM of Caspr3TM[20]. However, there is also a soluble form of the protein (CNTNAP3S or Caspr3S) that does not contain these exons, which expression has not been evaluated in the present study. In this sense, a mutation in the protein CNTNAP2 —other member of the NCP family— produces a soluble form of the protein that has been associated to mental pathologies as autism[53]. We cannot discard the possibility of changes associated to this protein form, since these family soluble forms have been associated to neuronal maturation and migration[53], processes altered in schizophrenia[54].

6.2. Antipsychotics Treatments: Concentration-Dependent and Time-Dependent Expression

We have focused this study in the short-term effects of different concentrations and time-points of four antipsychotics on CNTNAP3 gene expression. Results show that CNTNAP3 gene expression increases after treatment with the FGA haloperidol and the SGAs clozapine and risperidone, while the TGA aripiprazole produces a decrease on CNTNAP3 expression in a dose-dependent manner. A possible explanation for these different results could be due to the different mechanisms of action of first, second and third generation antipsychotics. Both first and second generation antipsychotics act as dopaminergic D_2 receptor antagonists, while the third generation antipsychotics act as partial agonists on this receptor (Table 1)[10]. In this sense, risperidone presents higher D_2 receptor affinity compared to clozapine[10], which can be correlated to the lower risperidone concentration needed in our experiments to obtain changes in CNTNAP3 gene expression. Similar results were obtained in the time-dependent gene expression experiments, with an increased CNTNAP3 gene expression for FGA and SGA, at least in the first hours, and a decrease for TGA. This D_2-mediated effect could explain the opposite effect of FGA and SGA compared to TGA. However, it would be needed further

experiments to check this possibility, studying the modulation of CNTNAP3 expression using different specific D_2 agonists or antagonists.

The different pattern in CNTNAP3 expression promoted by the different antipsychotics over time can be associated to different patterns, as reported for different genes modulated by haloperidol treatment[55]. They correspond to a continuous gene expression increase, as observed for haloperidol, an initial expression increase that returns to basal levels after 8 hours (clozapine and risperidone), and an initial expression decrease, that goes back to initial expression values after 8 hours (aripiprazole).

As the changes induced by the antipsychotic treatments in the neuroblastoma cell line reported in this study are not clearly a downregulation of CNTNAP3 expression, especially in the case of first and second generation antipsychotics, another possible explanation could depend on long-term effects of antipsychotics. Although the idea of a "delayed onset" –the *depolarization block theory*[31-] of antipsychotic action has been refuted in the last years[56,57] (suggesting that the repeated antidopaminergic effect on dopaminergic neurons in the brain results in a decrease of dopamine neuron firing and dopamine turnover which takes around 3 weeks), it has been proven that full therapeutic effects takes some time (weeks or even months) where patients can still suffer from part of the symptoms[57]. Thus, our 24-hour study shows a short-term gene expression response to the antipsychotic drug treatments, which can difficultly be compared to the 3-month investigation on the initial study of Crespo-Facorro[19]. Therefore, expression changes after chronic antipsychotic treatment could be due to compensatory changes promoted by the antipsychotic treatment instead of a direct effect. In addition, this differential effect could also depend on the toxicity elicited by antipsychotic drugs, as has been described for drugs as haloperidol[58].

Another explanation could be due to the different origin of the samples used in both studies: blood samples from treated and untreated schizophrenia patients, versus a neuroblastoma cell line. As is known, changes in gene expression induced by antipsychotic treatments are different depending on the brain area[59,60]. In this sense,

the downregulation of CNTNAP3 induced by different antipsychotics has been measured in plasma samples of psychotic patients[19], which could account for the differences obtained in the neuroblastoma cell line treatments.

Furthermore, the initial study was performed in psychotic patients that presented an upregulation of CNTNAP3 gene expression[11,19]. In contrast, the cell line used in this study, which does not represent a model for schizophrenia, may have a relative low basal CNTNAP3 gene expression. This could suggest that the normalization of CNTNAP3 expression after antipsychotic treatment found in previous studies with schizophrenic patients is only produced when there is an initial dysregulation of CNTNAP3 gene expression.

Finally, heterogeneity in our results can be also explained by CNTNAP3 biological role. In this sense, CNTNAP3 is the only member of the NCP family expressed in oligodendrocytes[20], with an important role in connection between neurons and glial cells. This could be correlated to the changes observed in schizophrenia, due to oligodendrocyte and myelin dysfunction that results in the impairment of synapse formation, function, and connectivity between different brain regions. This reduction in fine-tuning within the brain eventually leads to the cognitive dysfunction observed in schizophrenia[61]. Therefore, it would be of interest to repeat these studies in a mixed neuron-glia cell culture[62].

Another possible appropriate method is the use of a cellular model of schizophrenia, as the human induced pluripotent stem cells (hiPSCs)[63]. This neurons have shown diminished neuronal connectivity, decreased number of neurites and impaired synaptic maturation and, what is more important, gene expression studies with hiPSC neurons allow to compare antipsychotic treatments on live patients with clinical treatment eliminating confounding variables of postmortem analysis and offering alive schizophrenia cells[63].

6.3. LIMITATIONS AND FUTURE RESEARCH

We are aware of the limitations of the present study, which have to do mainly with time restrictions. More samples would be needed, especially in the case of concentration-dependent expression. Furthermore, longer treatments would be necessary to take into account possible toxic or compensatory effects. In addition, a comparison with the remaining five genes from the initial study[19] would have been interesting in order to analyze similarities and differences.

We think further investigation is needed to solve these limitations and to obtain more conclusive results, by using neuron-glial cell lines or human induced pluripotent stem cells, treated with longer period times and using more samples.

In addition, we believe more research is necessary to examine the involvement of the dopamine D_2 receptor as possibly responsible for the antipsychotic-mediated changes in CNTNAP3 gene expression.

7. CONCLUSIONS

- ✓ The expression pattern in SK-N-SH and HeLa cell lines was similar for ADAMTS2, ANTPD2, RFX2 and UNC45B genes. CD177 and CNTNAP3 genes presented a differential expression in both cell lines.

- ✓ CNTNAP3 gene expression was increased in a concentration-dependent manner after treatment with haloperidol (FGA), and clozapine and risperidone (SGA), while aripiprazole (TGA) produced a reduction of its expression after 24 hours.

- ✓ CNTNAP3 showed a differential time-dependent expression:
  - Constant increase in the first 24 hours for haloperidol.
  - An initial expression increase in the first 8 hours for clozapine and risperidone.
  - An initial expression decrease in the first 8 hours for aripiprazole.

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