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Caffeine Effects on the Gene Expression Profile of iPSC-derived Neuronal Cells

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

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UNIVERSIDADE NOVA DE LISBOA

Setembro de 2017

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Acknowledgements

To start with, I would like to thank my supervisor, Carsten Daub, for giving me the opportunity to develop this project and to take part in other scientific projects as well. For always supporting and encourage my work, for all the scientific critics and suggestions that allowed me to learn so much.

To my dear group colleagues that, one way or the other, contributed to my work and to lots of enjoyable and fun moments that I will always remember. A special thanks to Enrichetta, Kadir and Matthias that closely supervised my work. You were always willing to help me, professionally and personally. As I once wrote somewhere special, thank you for helping me to grow.

To Charlotte and Paul, for teaching me new techniques and always giving great support. To Andrea and Isabel for the opportunity to increase my work experience in the lab and for scientific feedback.

Quero agradecer aos meus pais, pelo vosso apoio incondicional sem o qual esta experiência não teria sido possível. Por todo o apoio e dedicação que sempre me deram, pela motivação quando faltava e pela apreciação que sempre demonstraram face ao meu trabalho. Ao meu irmão, meu companheiro desde sempre e que sempre me apoiou, apesar de todos os típicos arrufos de irmãos. Quero também agradecer a toda a minha família que nunca deixou de mostrar o quanto acreditam em mim, aos meus avós, aos meus tios e aos meus primos, pelo amor, carinho e dedicação que sempre me deram.

Quero agradecer ao Diogo, pelo apoio e motivação que sempre me deste, pela paciência com que aturas as minhas crises de mau-feitio e por saberes sempre como acalmá-las. Por me incentivares a querer aprender sempre mais e a aceitar novos desafios.

Aos meus amigos que sempre tiveram uma palavra de encorajamento e de conforto e que, de uma maneira ou outra, contribuíram para este meu percurso académico e pessoal. Um agradecimento especial à Rita, amiga, companheira, colega de casa que, com muita paciência, esteve sempre presente, sempre disposta a ajudar e que me acompanhou nesta aventura. E que grande aventura foi.

Resumo

A cafeina é consumida em grandes quantidades ampla e regularmente sob a forma de café, chá ou bebidas energéticas. Esta substância psicoactiva e muito popular é muitas vezes procurada devido à sua capacidade de aumentar a sensação de energia e estado de alerta de quem a consome, assim como o desempenho físico e cognitivo. Tem, igualmente, uma importante contribuição no aumento da capacidade de concentração. Estudos anteriores têm vindo a investigar a capacidade de baixas doses de cafeína para inibir a activação dos receptores de adenosina, bem como diversos outros efeitos farmacológicos incluindo o seu potencial em vários estudos de doenças neurodegenerativas. No entanto, pouco é conhecido relativamente aos efeitos da cafeína ao nível da expressão génica em células neuronais.

Neste estudo, pretendemos compreender se e como concentrações fisiológicas de cafeína podem afectar a expressão génica em células neuronais derivadas de células estaminais pluripotentes induzidas humanas. Mais ainda, identificamos *enhancers* activos em células neuronais e investigamos em que medida estes podem estar envolvidos na regulação da resposta neuronal à cafeína.

Por intermédio da utilização de uma técnica de análise do perfil de expressão de RNA – *Cap Analysis of Gene Expression* (CAGE) – obtivemos um conjunto abrangente de dados com respeito a regiões de início de transcrição e actividade em regiões de *enhancer* em células neuronais expostas a diferentes concentrações de cafeína (0, 3 e 10 µM).

Identificámos um conjunto de genes que aparenta estar envolvido na mediação da resposta à cafeína. Após 1 hora de tratamento com 3 µM de cafeína ocorre um aumento de actividade sináptica, bem como de neurotransmissão dopaminérgica. Processos do sistema imunológico, assim como a projecção axonal encontram-se reprimidos após 3 horas de exposição a 10 µM de cafeína.

Estes resultados levantam hipóteses concretas sobre processos fisiológicos e genes a eles associados que podem orientar futuros trabalhos experimentais com fim a validação funcional que, por sua vez, pode conduzir a novas descobertas respeitantes ao efeito da cafeína no cérebro humano.

Palavras-Chave

Cafeína, Receptores de Adenosina, Expressão Génica Diferencial, *Cap Analysis of Gene Expression* (CAGE), *Enhancer*, Transcriptoma

Abstract

Caffeine is widely and massively consumed on daily basis in the form of coffee, tea or energy drinks. This very popular, psychoactive drug is sought after due to its ability to increase energy and alertness, enhance physical and cognitive performance as well as to improve our ability of focusing. Previous studies have investigated the ability of caffeine to inhibit the activation of adenosine receptors in low doses, amongst other pharmacological effects as well as its potential in several neurodegenerative disease-modeling studies. However, not much is known about the effects that caffeine exerts on gene expression of neuronal cells.

In this study we aim to understand if and how physiological concentrations of caffeine affect gene expression in human induced pluripotent stem cell (iPSC)-derived neuronal cells. Moreover, we identify active enhancers in neuronal cells and investigate the extent to which enhancers might be involved in the regulation of neuronal responses to caffeine.

Using Cap Analysis of Gene Expression (CAGE) RNA expression profiling, we obtained a comprehensive data set of transcription start sites and enhancer activity of neuronal cells exposed to various caffeine concentrations (0, 3 and 10 µM).

We identified a set of genes that appears to be involved in the mediation of caffeine response. Synaptic activity is upregulated after 1 hour of 3 µM caffeine treatment as well as dopaminergic neurotransmission. Immune system processes as well as axon guidance events are downregulated after 3 hours of 10 µM caffeine exposure.

These insights provide concrete hypotheses of physiological processes and associated genes for guiding further functional validation experiments with the potential to give valuable insights into the effects of caffeine to the human brain.

Keywords

Caffeine, Adenosine Receptor, Differential Gene Expression, Cap Analysis of Gene Expression (CAGE), Enhancer, Transcriptome

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List of Abbreviations

CAGE – Cap Analysis of Gene Expression

Lt-NES cells – Long-term self-renewing neuroepithelial-like stem cells

iPSC – Induced pluripotent stem cell

TC – Tag cluster

CPM – Counts per million

TSS – Transcription start site

CTSS – CAGE-defined transcription start site

FDR – False discovery rate

GLM – Generalized linear model

GO – Gene ontology

NGS – Next Generation Sequencing

PCA – Principal component analysis

RLE – Relative log expression

1. Introduction

Coffee and Tea – Impact on Society and Economy

Caffeine is widely and massively consumed worldwide. Coffee, tea and energy drinks are three of the most popular forms of caffeine consumption, with coffee in first place. Chocolate bars and soft drinks are also a dietary source of caffeine, especially for children (Lorist and Tops, 2003; Fredholm *et al*., 1999). Caffeine pills are also one way of caffeine consumption, usually used for cognitive enhancement. Figure 1.1 displays the caffeine content, in milligrams, of common dietary sources of caffeine, according to the European Food Safety Authority (European Food Safety Authority, 2015). Two studies have approached German university students' consumption of caffeine pills, accounting for 10.5% students stating that they used caffeine pills at least once in their lifetime in one study (Franke *et al.*, 2011) and 13.4% in other study (Brand and Koch, 2016). According to Food and Drug Administration (FDA), caffeine pills can have a caffeine content of 100-200 mg per tablet/capsule.

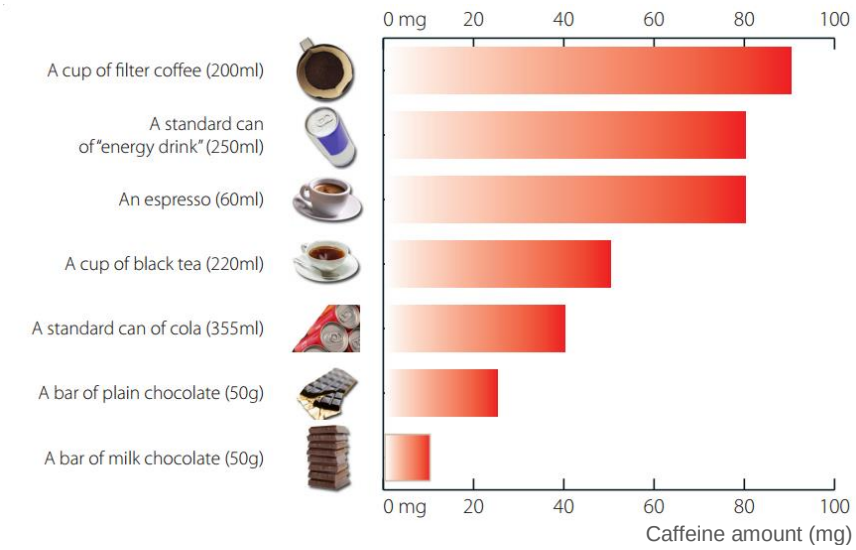

Figure 1.1 – Amount of caffeine found in common dietary sources of caffeine. Graph demonstrating the amount of caffeine, in milligrams (mg) that can be found in several dietary sources, such as coffee, tea and chocolate.

Coffee is part of social interaction nowadays. People get together over coffee on their free time, as well as during working hours as a way to have a pause (Lorist and Tops, 2003). Figure 1.2 illustrates the average number of coffee cups consumed by one person daily for the top 10 countries that consume most coffee in Europe (Statista Consumer Market Outlook, 2015). With an average of 3.6 cups of coffee ingested by one person daily, Finland leads the top 10, followed by its neighbors Sweden, Netherlands and Denmark with 2.9, 2.8 and 2.4 cups of coffee, respectively. Portugal consumes an average of 1.3 cups of coffee per day, side by side with France. Brazil and North America are also pronounced coffee consumers worldwide, with an average of 1.6 and 1.2, respectively.

The levels of caffeine consumption through coffee intake are the reason why studies on the caffeine effects are so important, so needed and received with great interest by the population. Whether these studies address short term effect or long term effects, all information regarding the effects of caffeine on the human body, particularly the human brain, are of great interest for the majority of the population that more or less consumes caffeine.

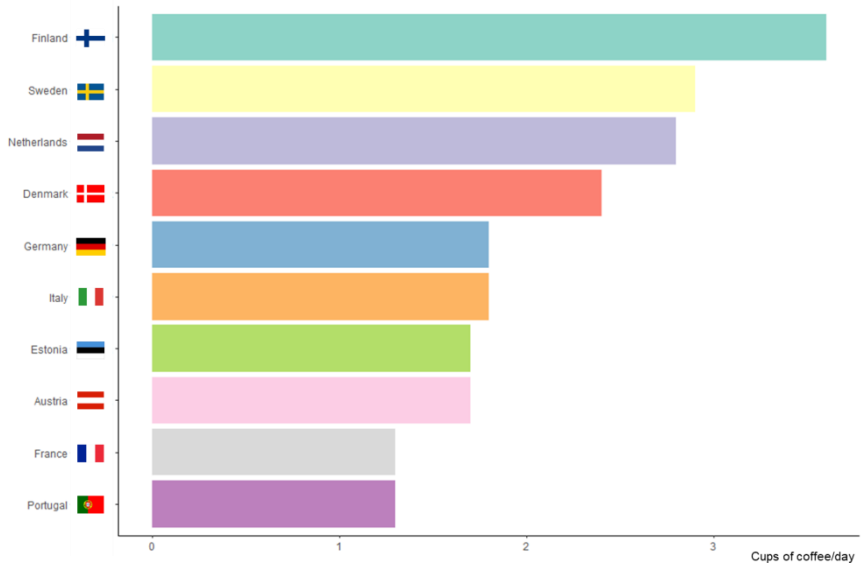

Figure 1.2 – European countries with highest rates of coffee consumption per capita. Illustration of the number of cups of coffee ingested daily per capita, on average, according to the Statista Consumer Market Outlook from 2015.

Caffeine

Caffeine – a methylxanthine – is a plant alkaloid and a very popular psychoactive drug (Glade *et al*, 2010; Fisone *et al*, 2003). People appreciate the psychostimulant effects of caffeine and the fact that there are no negative side effects documented so far, for physiological doses of caffeine. The ability of caffeine to enhance motor activity is one of the pronounced effects, as caffeine acts in regions of the brain related to motor activity (Fisone *et al*, 2003). Moreover, moderate doses of caffeine can increase energy availability and daily energy expenditure, which is related to enhancement of motor activity, decrease fatigue and the sense of effort associated with physical activity. Caffeine enhances cognitive performance, increases alertness, wakefulness and feeling of "energy". This compound also has the ability to decrease mental fatigue, accelerate and increase the accuracy of reactions, increase the ability to concentrate and focus attention, enhance short-term memory and the ability to solve problems that require reasoning as well as increase the ability to make correct decisions and enhance cognitive functioning and neuromuscular coordination (Glade *et al*, 2010). All of these responses constitute the behavioral effects of caffeine and can be measured and characterized. Commonly, variations in motor activity are the behavioral outcome of choice when quantifying the stimulant properties of caffeine.

Caffeine Absorption, Distribution and Metabolism

Upon ingestion, caffeine is almost completely (99%) and rapidly absorbed – 45 minutes upon ingestion – in the gastrointestinal tract and distributed in the blood stream reaching the whole body and passing through all biological membranes, such as the blood-brain barrier, due to its lipophilic properties (Liguori *et al*., 1997; Bonati *et al*., 1982). Between 15 and 120 minutes upon ingestion, caffeine concentration in plasma reaches peak levels (Arnaud, 1987).

Caffeine is metabolized in the liver and easily cleared from the body, presenting a half-life approximately between 3 and 5 hours (Grant *et al.,* 1987; Fisone et al, 2003; Lorist and Tops, 2003). The clearance rate of caffeine from the body can be affected by several compounds, such as nicotine or oral contraceptives (Lorist and Tops, 2003).

Pharmacological Effects of Caffeine

As most methylxanthines, caffeine has a very similar structure to cyclic nucleotides and can, therefore, easily interact with cyclic nucleotides phosphodiesterases. However, significant effects are only achieved with concentrations in the millimolar range since caffeine affinity for phosphodiesterases is low (Fisone et al, 2003). Other studies showed that caffeine inhibits the γ-aminobutyric acid (GABA) receptor for concentration in the 350-500 μM range (Fisone et al, 2003).

The fact that, even with high concentrations of caffeine and a high absorption rate, only a small part is able to reach intracellular targets, such as phosphodiesterases or GABA receptors, is important to consider. This means that measuring the effect only on the level of these intracellular targets is not entirely illustrative of the effects of the concentration in study (Fisone et al, 2003). Caffeine has also been shown to modulate CREB-dependent gene expression and to induce the expression of immediate early response genes (Svenningsson *et al*., 1995; Connolly and Kingsbury, 2010).

Caffeine modulation of neurotransmission in the brain is mainly dependent on its ability to act as antagonist of adenosine receptors. Adenosine, a purine compound, is a neuromodulator that, generally, inhibits neuronal activity. Although it clearly has an effect in the central nervous system neurotransmission processes, adenosine is not considered a neurotransmitter since it does not fulfill some of the current criteria of neurotransmitter definition; it is not accumulated into vesicles and it is not released in synapses in a calcium-dependent manner like all neurotransmitters (Fisone et al, 2003).

Adenosine activity depends on the presence of its receptors in various regions of the brain. Four receptors have been described so far – A_1, A_{2A}, A_{2B} and A_3 – and are expressed in the brain. However, receptors A_{2B} and A_3 have low levels of affinity for adenosine and have only basal levels of activation. Caffeine effects do not include inhibition of these two receptors. On the other hand, adenosine receptors A_1 and A_{2A} have high affinity to adenosine/caffeine and can be activated by nanomolar concentrations of adenosine, which is the normal range present in the brain (Fisone et al, 2003; Lorist and Tops, 2003) and, thus, caffeine effects are mediated by inhibition of the latter two receptors.

Unlike the majority of mechanisms by which caffeine has its effect on the brain, blockade of adenosine receptors is the only one that can be triggered by lower concentrations of caffeine (Lorist and Tops, 2003). Thus, caffeine consumption that derives from a regular and daily ingestion of coffee or tea should not be expected to activate other mechanism rather than inhibition of adenosine receptors.

Adenosine – Mechanism of Action

Neurons, like every other cell type, use ATP as a source of energy for most cell processes. In order for the energy to be available, ATP has to be broken down. When this happens, adenosine is released as a break down sub product and its intracellular concentration increases in neurons. Part of this intracellular adenosine leaves the neurons and binds to adenosine receptors through specific membrane transporters (Fredholm *et al.*, 1999). The effect of adenosine binding to its receptors can be excitatory or inhibitory, depending on which receptor is activated. As mentioned above, adenosine activity is mediated mainly by A_1 and A_{2A} receptors. A_1 receptor is highly expressed in neurons that stimulate awakeness, whereas A_{2A} receptors are mostly found in neurons that promote sleeping.

Adenosine acts on A_1 receptors by inhibiting them and on A_{2A} receptors by activating them (Fredholm *et al*., 1999). Intense brain activity means neurons used a lot of ATP to provide for energy and the levels of adenosine are very high. High levels of adenosine binding to A_1 receptors decreases activity of wake-stimulating neurons and high levels of adenosine binding to A_{2A} receptors increase the activity of sleep-promoting neurons. Therefore, adenosine decreases brain activity and induces sleep, preventing the brain from overworking. Later, during sleep, adenosine is gradually eliminated until there is not enough to activate the receptors and activity restarts.

Caffeine, with a similar structure to adenosine (Figure 1.3 and Figure 1.4), can also bind to adenosine receptors. However, instead of activating them, caffeine acts by competing with adenosine for the receptors and blocks the receptors so that adenosine cannot bind efficiently until caffeine is cleared after a period of 2-4 hours, depending on the metabolism rates of the organism.

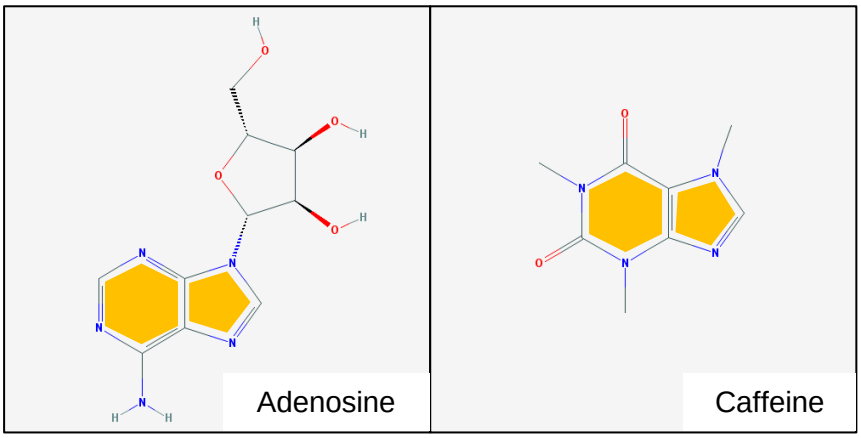

Figure 1.3 – Adenosine and caffeine structure similarities. On the left panel, an illustration of adenosine chemical structure. On the right panel, an illustration of caffeine chemical structure. Highlighted in yellow are the common features of both structures responsible for the ability of both molecules to bind to adenosine receptors. Figure adapted from PubChem Compound Database.

Caffeine is not considered as addictive and does not present negative side effects, as mentioned earlier. However, a mechanism known as caffeine tolerance is developed over long periods of caffeine consumption. When caffeine is ingested continuously, neurons start to increase the synthesis of adenosine receptors and more caffeine is needed so that the same effect is attained. When caffeine ingestion stops abruptly, adenosine effect is higher due to a higher number of receptors being activated and sleep induction intensifies. This leads to drowsiness, irritability, fatigue and difficulties in concentrating. To reverse these unpleasant symptoms, people keep consuming caffeine leading to a mild physical addition to caffeine, but not severe enough to be classified as a real addiction.

Other organs express adenosine receptors, which is the case of the heart and kidneys. Both these organs express A_1 receptors and binding of these receptors in heart and kidney has the same effect as in the brain – decreasing the activity of the cells that express them. Caffeine acts as an antagonist in these organs as well increasing the heart rate and the amount of blood filtered by the kidneys and, thus, increasing urine production. Subjects who ingest high doses of caffeine can experience headache, dizziness and insomnia due to over-alertness, jitteriness due to higher heart rate and dehydration from increased urine production.

The effects of caffeine on a developing brain are not well known and, because of this, it is important to moderate caffeine ingestion by children.

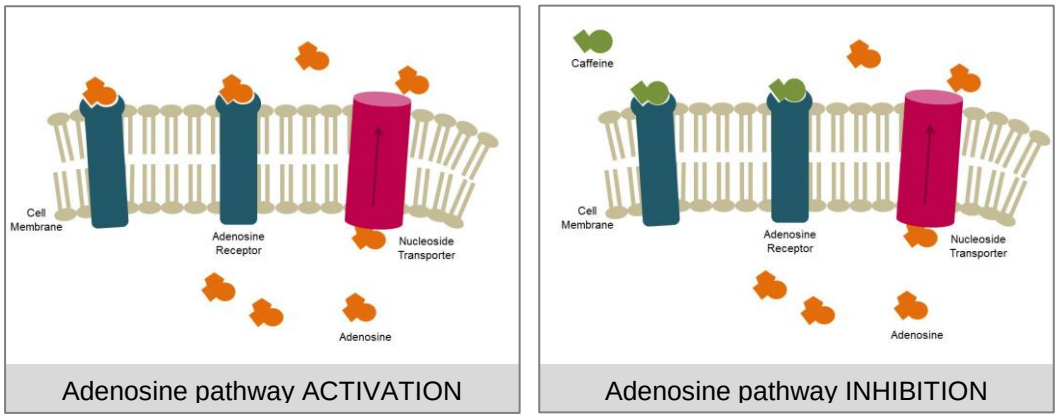

Figure 1.4 – Adenosine mechanism of action and caffeine perturbation of this mechanism. On the left panel, activation of adenosine receptors by adenosine binding, in absence of caffeine. On the right panel, inhibition of adenosine receptors by antagonist binding of adenosine receptors by caffeine instead of adenosine.

iPSC-derived Neuronal Cells – NES Cells

Studies on neuronal cells are, often, conducted using animal models or immortalized cells lines such as SH-SY5Y cell line. Although these models can give valuable insights, the results are always difficult to translate into the human correspondent parts (Dolmetsch and Geschwind, 2011; Dragnunow, 2008). However, so far and concerning the human brain, they have been the best option. A new alternative that promises to change how we conduct studies involving neuronal cells or even studies on neurological diseases appeared with the development of cell reprogramming techniques (Dolmetsch and Geschwind, 2011). Pluripotent state can nowadays be easily induced from human somatic cells, such as human fibroblasts, and cells can be reprogrammed to differentiate into different cell types from the original. Reprogramming techniques include the introduction of reprogramming factors that, generally, consist of transcription factors, such as Oct4, Sox2, klf4, c-myc, Nanog and lin28 in the cells that we want to dedifferentiate (Takahashi and Yamanaka, 2006). iPSCs have the ability of self-renewal in culture and of generation of most cell types in the human body. When

induced pluripotent stem cells are exposed to the adequate combination of growth factors and culture conditions it is possible to induce differentiation into neuronal cells, or several other differentiated cell types (Masui *et al*., 2007). Induced pluripotent stem cell (iPSC) differentiation into neuronal cells has the potential to overcome the gap between human neuronal studies and the model organisms used to address them. Previously unattainable, iPSC's allow the study of human neurons that carry a specific mutation, which is the case of several neurodegenerative diseases; or simply develop studies on healthy human neurons, which is the case of the present study.

Falk *et al* developed a protocol to obtain long-term self-renewing neuroepithelial-like stem cells (lt-NES cells) from induced pluripotent stem cells and, posteriorly, differentiate these into neuronal cells, (Falk *et al*, 20012). This cell line can overcome ethical problems raised by embryonic stem cell use and obstacles imposed by previously mentioned animal models or immortalized cell lines. It is a healthy human cell line that provides a mixture of neurons and glial cells, presenting good and improved modeling of the human brain. Lt-NES cells are a step towards better modeling of neurodegenerative diseases that affect neurons and glial cells or neurotoxicity studies (Falk *et al*, 2012).

In the present study, lt-NES cells were differentiated into a mixture of neurons and glial cells used to assess the response, on gene expression level, when exposing cells to physiological concentrations of caffeine. Similarly to other studies involving neuronal cells, studies on the effects of caffeine in neuronal cells, including neurotoxicity studies, have also been conducted either in animal models or immortalized cell lines, such as SH-SY5Y. Through the use of lt-NES cells-derived neurons and glia cells, the results obtained are more reliable and close to the real response of healthy human neurons or glial cells.

Gene Regulation and Chromatin Conformation

All somatic cells share the same DNA; however the selective expression of each cell genome confers cell type specificity. Expression of different sets of genes in different cell types depends on several mechanisms of gene regulation that can happen on many levels. Chromatin accessibility can be regulated to ease or difficult gene expression, by changes in the chromatin structure that facilitate or constrain the access of transcription machinery to specific regions of the genome. Different combinations of transcription factors and proximal and distal regulator regions are responsible for transcription of different sets of genes. mRNA molecules undergo processing steps that can regulate their final expression. Translation of these molecules into proteins might be increased or decreased and the resulting proteins can also suffer modifications that will implicate the final function. From gene expression to phenotype expression several are the regulation steps where the fate of a protein or functional RNA can be reformed. Gene regulation not only is the foundation of cell type specificity, but is also the reason for differences between species.

State of chromatin conformation interferes with promoter's availability to be accessed by the transcription machinery. DNA looping is one way of chromatin remodeling that consists in physical interaction of two distant genomic regions that force a loop in the DNA. The combination of several regulatory mechanisms leads to the relaxation of chromatin structure in both regions and the motifs can interact. This happens when regulatory regions such as enhancers regulate their target promoters (Calo and Wysocka, 2013).

Transcribed Enhancers

Enhancers, short DNA regions (50-1500 bp), are key regulatory elements of transcription that act towards promotion of transcription initiation by binding transcription factors that in turn interact with other transcription factors bound to promoter regions. Enhancers can be located up- or downstream of the promoters they interact with and they act independent of strand orientation (Maston *et al*., 2006; Stees *et al*., 2012). Downstream enhancers can be located within introns or beyond the 3' end of the target gene (Visel *et al*., 2009). Similarly to promoter regions, enhancers are constituted by modules, which allows for activation of promoters in a time- and tissue-specific manner (Maston *et al*., 2006). Enhancers are cis-acting regulators, acting on promoters located in the same DNA molecule, but not necessarily on the closest promoter (Andersson *et al*., 2014) and can be several hundred kilo base pairs distant from the target promoter (Visel *et al*., 2009).

Recent development in transcriptomics and genomics endorsed the acknowledgement of the importance of regulatory elements that far outnumber protein-coding genes (ENCODE Project Consortium, 2012). These regulatory elements have been gradually associated with several human diseases and, thus, deep understanding of the mechanisms that underlie gene regulation is very important (Visel *et al.,* 2009; Li *et al.*, 2016).

Protein-protein interactions between enhancer regions and target promoters as well as DNA-looping were, for a long time, the explanative models for transcription regulation by enhancers (Heintzman et al., 2009a). However, studies showed that RNA polymerase II is also found in non-promoter regions such as enhancers, which indicates active transcription of enhancers. (Koch *et al*., 2008; Li *et al*., 2016). RNA sequencing showed that enhancers are transcribed in a bidirectional fashion originating enhancer RNA (eRNA) (Kim *et al*., 2010; De Santa *et al.*, 2010; Djebali *et al*., 2012; Andersson *et al*., 2014). Various genomic features distinguish active enhancers and poised enhancers. Binding of enhancer regions by p300 and monomethylation of H3K4 in flanking nucleosomes are representative of poised enhancers and recruitment of RNA polymerase II, production of eRNAs and acetylation of H3K27 are associated with active enhancers (Heintzman *et al*., 2009b; Kim *et al*., 2010, Wang *et al*., 2011).

Contribution of gene expression regulation by enhancers is certain and well characterized. However, the mechanisms by which enhancers regulate gene expression still remain poorly understood. eRNA

expression has been confirmed to occur in many cell types and is considered an universal gene expression regulation mechanism (Kim *et al*., 2015). Whether and how transcriptional activity at enhancers contributes to enhancer function is still an open question. Studies indicate that eRNA synthesis occurs only when the enhancer is interacting with the target promoter (Kim *et al*., 2010; Lai *et al*., 2013). eRNA activity on specific target promoter might be achieved due to its high instability suggesting eRNA exert their function in the target promoter and not in other promoters located away from the local of synthesis (Kim *et al*., 2015).

Cap Analysis of Gene Expression (CAGE)

Cap Analysis of Gene Expression (CAGE) is a high throughput sequencing technique that allows screening the transcriptome of cells and quantitatively detect gene expression on the promoter level. The technique is based on several sequencing techniques and it was developed by Piero Carninci and Yoshihide Hayashizaki's group at RIKEN Yokohama Institute in Japan. Like many other methods, CAGE counts with next generation sequencing (NGS) (Takahashi *et al.*, 2012a; Takahashi *et al*., 2012b; Shiraki *et al*., 2003; The FANTOM consortium and the RIKEN PMI and CLST (DGT), 2014).

CAGE uses a cap trapping method to isolate mRNAs from the total RNA extracted from cells. mRNA molecules are protected in the 5' end by a structure called CAP. This structure distinguishes mRNA molecules from the majority of other RNA molecules. During the first steps, random primers initiate the synthesis of a complementary cDNA strand that will extend up until the capped 5' end of the molecule or before that. Only the hybrids in which the cDNA strand reached the capped 5' end are kept after single strand RNA regions are digested with RNase. As a result of labelling all cap structures with biotin, mRNAs can be pulled with streptavidin beads and a library of all cDNAs derived from the 5' ends of transcripts is assembled using 5' end and 3' end sequencing primers and linkers. The libraries are sequenced and aligned to the genome. Since CAGE captures 5' ends of coding or non-coding RNAs, it means that when the reads are aligned to the genome they are mapping to transcription start sites (TSSs), regions of the genome where transcription is initiated. The amount of reads mapping to each TSS indicates the level of expression of each TSS and after associating each TSS with a specific gene, via genomic annotation, we know the level of expression of each gene. Since CAGE captures expression of TSSs and usually transcription is initiated in promoter regions, this method allows the study of alternative promoters as well as assessing which transcription factors are involved in specific transcription initiation.

CAGE is one of several RNA sequencing techniques, focusing on the expression of TSSs by cap trapping the 5' ends of mRNA transcripts. RNA sequencing techniques were developed as an alternative to the previous gold standard methods, array-based assays. The first obvious advantage of CAGE over RNA micro arrays is that CAGE allows a genome wide assay, whereas array-based techniques can only detect as many transcripts as the number of probes used in the array. Of course the number of probes does not extend to the total of genes in the human genome. Detecting TSSs or

promoters' expression is a characteristic advantage that CAGE shows over the rest of RNA sequencing techniques as well as array-based methods.

CAGE uniqueness relies on its ability to detect transcription start sites at a single-nucleotide resolution level, as no other RNA-sequencing technique can. CAGE only sequences small tags of cDNA corresponding to the 5' end of mRNAs, which means that lower amounts of sequencing are needed, compared to RNA-seq techniques.

CAGE can also detect expression of enhancers. Expressed enhancers have bidirectional and bimodal expression which means that transcription is initiated in both strands, forward and reverse (Andersson *et al*., 2014; Arner *et al*., 2015). With this in mind it is possible to identify expression of enhancer regions, even though enhancers are very lowly expressed. CAGE has detected 40 000 - 65 000 eRNAs, representing a large part of transcription initiation events in the human transcriptome (Andersson *et al*., 2014). An approach similar to the one described by the latter study allows a categorization of enhancers in cell-specific and ubiquitous enhancers, assessment of interactions between enhancers and promoter regions as well as identification of single nucleotide polymorphisms (SNP) potentially associated with human diseases.

The FANTOM5 project established an atlas of CAGE-defined TSSs (Andersson *et al*., 2014). Andersson *et al.* demonstrated that bidirectional transcription is a mark for active enhancers (Andersson *et al*., 2014). A simple illustration of sequential steps that follow CAGE library preparation is depicted in Figure 5.1.

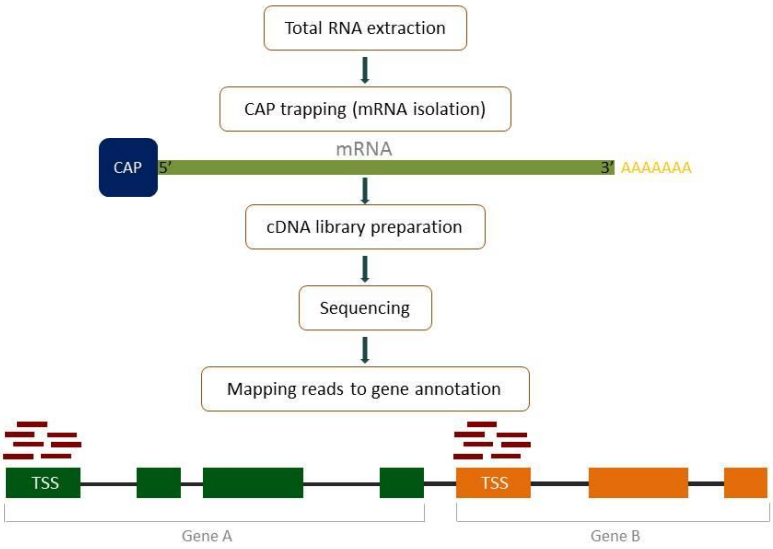

Figure 1.5 – Illustration of the sequential steps of CAGE library preparation and data processing.

2. Project Aims and Hypothesis

Previous studies mainly reported pharmacological targets of caffeine, such as adenosine receptors. No preceding studies have reported effects on gene expression levels in neuronal cells using iPSC-derived neuronal cells as model and in a genome-wide fashion. Contrary to other caffeine mechanisms of action, blockade of adenosine receptors can be triggered by lower concentrations of caffeine. Thus, we assume that caffeine effect is mediated, even if in part, by changes in gene expression that occur, presumably, downstream of adenosine receptors mechanism.

In this project, we address three main questions:

1. Identification of genes mediating caffeine response in iPSC-derived neuronal cells in different concentrations and during different times of exposure.

2. Identification of active enhancers in iPSC-derived neuronal cells.

3. Identification of enhancers that regulate the mediator genes identified in aim 1.

Although this work consists of bioinformatics analysis mainly, prior work was done in order to have the experimental data that allowed the analysis that culminated in the answers to the questions we proposed to answer. Cell reprogramming, neuronal differentiation and cap analysis of gene expression are the experimental methods chosen to address our questions and were performed prior to the work presented in this thesis.

Answering these questions can provide significant insights on how the human brain reacts to caffeine, particularly the nervous system. Even though caffeine is already well characterized when it comes to pharmacological effects in the nervous system, the same does not happen regarding the gene expression changes associated with these pharmacological effects. Deeper knowledge on gene expression changes in response to caffeine stimuli, resulting from deep transcriptome sequencing, can be very valuable and applied to specific studies on neurodegenerative diseases or toxicity, for example.

3. Materials

Cell Differentiation and Caffeine Exposure

Human long-term self-renewing neuroepithelial-like stem cells (NES cells) were obtained as described in *Falk et al* (2012) and then differentiated into neuronal cells for 38 days by removal of growth factors. Two batches of cells were treated with 3 µM and 10 µM of caffeine each and 1 batch was not treated with caffeine. Cells were collected, in replicates of 3 or 4, in the beginning of the experiment – 0 hours – and after 1, 3 and 9 hours. RNA was extracted using NucleoSpin RNA Kit (Macherey-Nagel), according to manufacturer's instructions. An illustration of the differentiation process is depicted in Figure 3.1.

The obtained cells are estimated to be a mixture of 80-90% neurons and 10-20% glial cells, according to immunofluorescence staining and bright field microscope imaging that confirmed the presence of these cell types (data not shown). The majority of the cell population showed clear expression of mature neuronal cell markers, such as MAP2 and NeuN.

This experimental procedure, illustrated in Figure 3.1, was performed before and is out of the scope of this thesis.

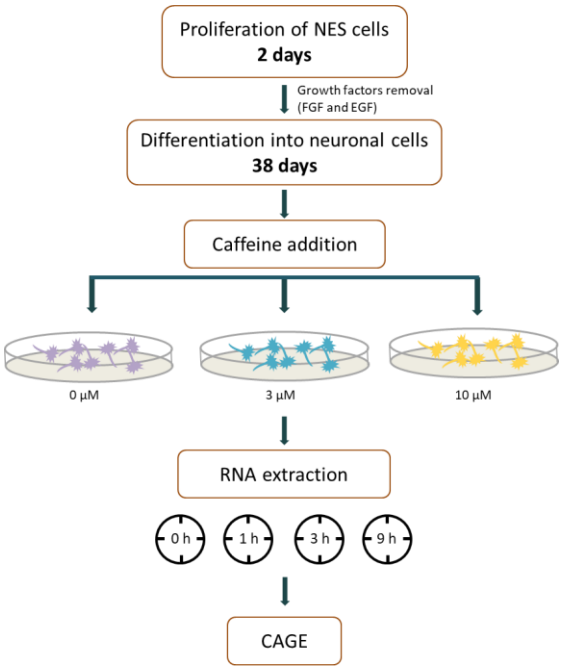

Figure 3.1 – Illustration of lt-NES cells differentiation process into neuronal cells and caffeine exposure. Proliferation of NES cells occurs during 2 days, followed by removal of growth factors. Differentiation into neuronal phenotype is triggered and lasts for 38 days. Cells are exposed to caffeine treatment (0, 3 and 10 µM) and RNA is collected from cells in the beginning of the experiment and after 0, 1, 3 and 9 hours of exposure.

CAGE Library Preparation

CAGE libraries were prepared with a total RNA input of 1 µg and 1% yeast (*Sacharomyces pombe*) spike-in for normalization, according to the original protocol (Takahashi *et al*., 2012b). Four libraries were prepared and pooled together for sequencing in Illumina Hi-Seq 2500 or 2000, in a randomized order to avoid batch effects. One of four random barcodes was attributed to each library and all four libraries were pooled together before sequencing. Barcoding of each library is achieved by using barcoded sequencing 5' end linkers.

These libraries were prepared before and out of the scope of this thesis.

Sequencing Data and Expression Table

Fastq files were provided as well as already processed data into a final table of expression counts for each tag cluster (TC), across all samples (see section 4, Experimental Design). Expression counts refer to the number of reads that align at a particular position. A tag cluster is a group of tags, or sequencing reads, that map on the same strand of a chromosome and overlap by at least 1 base pair (bp). Each TC represents a group of CAGE-defined TSS and, therefore, the expression table obtained after processing of the sequencing data provides, for each sample, the number of transcripts detected for each CAGE-defined TSS (Figure 3.2). All tag clusters were associated with the corresponding genes, using GENCODE v19 genome annotation.
The expression table was the starting point of the work presented in this thesis.

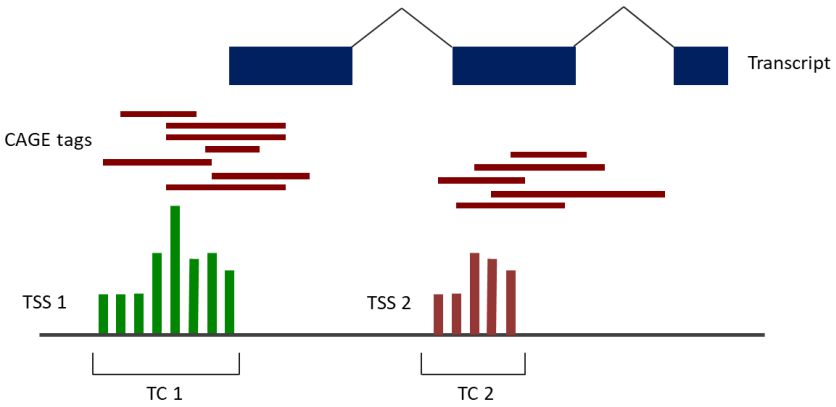

Figure 3.2 – Illustration of CAGE detection of alternative transcription start sites. TC 1 and TC 2 represent different tag clusters. Each CAGE tag represents one CAGE-defined transcription start site. CAGE tags are mapped to the TSS, the number of tags indicates the expression and a tag cluster is built based on overlapped mapping of CAGE tags.

4. Methods

Experimental Design

The design of the study was conceived in a way that different groups of cells are treated with two different concentrations of caffeine – 3 µM and 10 µM – or not treated. For each combination of concentration and time, three or four biological replicates were collected, as indicated in Figure 4.1. The control sample, encompassing three biological replicates, represents the cells from which RNA was extracted in the beginning of the experiment. These cells were not exposed to caffeine and the gene expression levels detected consolidate the normal pattern of expression expected from neuronal cells. The control sample is used as baseline/reference for all comparisons when assessing the effect of caffeine on gene expression. It is important to keep in mind that the cell model used consists of newly differentiated neurons and glial cells and, possibly, a fraction of still differentiating or non-differentiated cells.

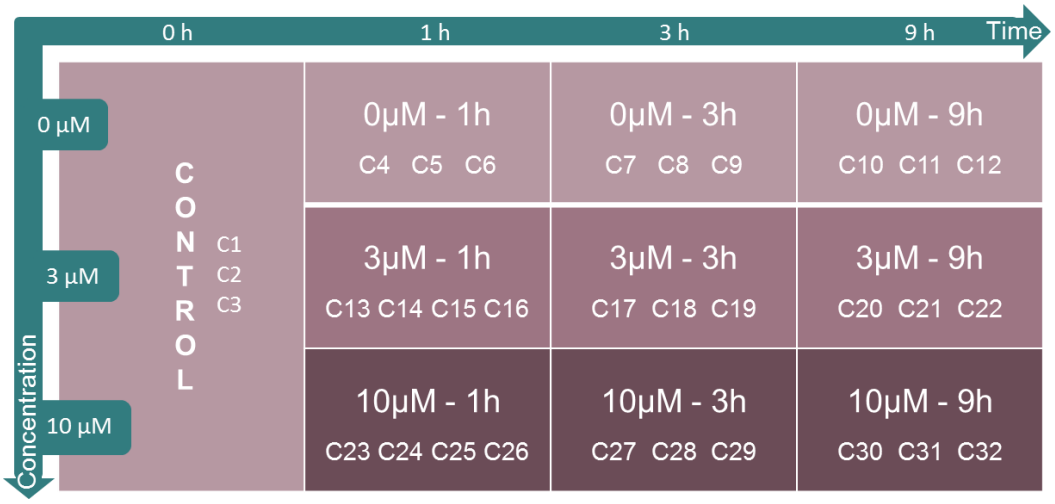

Figure 4.1 – Experimental design of the study. Representation of all samples studied, each one corresponding to a batch of cells exposed to different caffeine concentrations and different periods of time. C# represents the nomenclature attributed to the replicates collected from each sample.

Data Quality, Outliers and Sample Consistency

As mentioned before, the starting point of this work consists of a table with the number of transcripts detected at each tag cluster, for a total of 32 samples, representing 10 different experimental conditions. A big data set was created and 43,631 tag clusters were associated with their corresponding expression count.

Data was normalized using counts per million (CPM), for differences in library size, and relative log expression (RLE) that takes into account the size factor. To obtain the counts per million we scale the counts through division by the library size (total number of fragments detected) and multiplication by one million. The size factor used in RLE normalization is the ratio between tag cluster expression counts and the median expression for that tag cluster across all samples.

Lowly expressed TCs were removed from the analysis by applying an ad-hoc threshold of one CPM in at least two samples. Applying the CPM threshold means that only tag clusters that have two or more samples with expression higher than 1 CPM are chosen for further analysis. The 10 highest expressed TCs were also removed, as they were not found to be involved in caffeine response and represented housekeeping genes, micro RNA (miRNA) and long non-coding RNA (lncRNA). Since these genes have very high expression, they can alter normalization and influence downstream analysis.

As mentioned before, barcode randomization of samples was conducted prior to sequencing and batch effects associated to barcodes were assessed. For samples showing inconsistent expression levels between replicates of the same condition linked to barcoding, an extra filtering step was performed. Since the barcoding is random, replicates of the same condition can have different barcodes. A pattern was found in which for a specific barcode, several TCs had very high expression, while the equivalent replicates, with different barcode, had very low or no expression. Since this did not represent a barcode batch effect due to its isolated occurrence (68 in 43,631), this subset of TCs were removed from the following analysis.

Lastly, outliers were identified using visualization of distribution of normalized and not normalized expression across all samples. Sample C18 displays a different profile than the rest of the samples, presenting an abnormal number of TCs with very low or no expression, when compared to the two other replicates collected from the same condition. This was confirmed by looking at the number of TCs with no expression in all samples, from which C18 appears to be an outlier.

In order to confirm if C18 was in fact an outlier sample and if any other prominent outliers existed in the data set, principal component analysis (PCA) was performed as a dimension reduction method, before and after filtering the data.

Enhancer Prediction

For enhancer prediction, Andersson *et al*. unsupervised predictor was used. In brief, all the 31 CTSS files are combined into a unique file and split by strand. The CTSS files include all CAGE-defined transcription start sites (CTSS) detected in each sample. Decomposition-based peak identification (DPI) clustering algorithm is used to identify tag clusters from all the CTSS. The distance between peaks is set up using the *d* parameter. Expression is quantified, and enhancers are identified based on bidirectional expression. A mask file is used to remove enhancers that overlap with TSSs of known genes and exons (Andersson *et al*., 2014).

Lower *d* parameters result, usually, in better shaped enhancers, as the peaks included in the enhancer are closer together. Peaks that have a higher distance than *d* are not included in the prediction of one specific enhancer. *D* parameters of 5, 10 and 20 were tested and *d*=5 revealed the lowest number of enhancers, however better shaped (bimodal and bidirectional expression) and, thus, more reliable. Tag clusters englobing only one CAGE peak, were removed.

Gene Expression Responses to Caffeine Treatment

Differential gene expression was performed in R using the generalized linear model (GLM) implemented in edgeR package 3.14.0 (Robinson *et al*., 2010). All the comparisons made can be found in Table 4.1, with highlight on the ones used for the analysis reported in this thesis. The outline of the comparisons made and how the different results were integrated for one single analysis is shown in Figure 4.2. A cutoff of 0.1 FDR was used for differential expression significance level. No cutoff was applied for the fold change (FC), meaning that any value indicating up- or downregulation was reported.

Differentially expressed genes detected in both treated and non-treated cells were selected according to the difference between the fold change (log_2FC) levels in each condition. A threshold of 0.5 in log_2FC difference was applied to differentiate genes changes due to sample handling and gene changes in response to caffeine addition to the medium.

Table 4.1 – Comparisons used in differential gene expression analysis. All comparisons used in the analysis are listed associating two samples, each one consisting of a combination of a concentration and a time of exposure. A description of the meaning of the results retrieved from each comparison is shown.

Condition comparison	Description
0µM - 1h | CONTROL	Gene expression changes in **non-treated** cells after a period of 1, 3 and 9 hours
0µM - 3h | CONTROL	
0µM - 9h | CONTROL	
3µM - 1h | CONTROL	Time-dependent effect of caffeine in gene expression of cells treated with **3 µM** of caffeine after a period of 1, 3 and 9 hours
3µM - 3h | CONTROL	
3µM - 9h | CONTROL	
10µM - 1h | CONTROL	Time-dependent effect of caffeine in gene expression of cells treated with **10 µM** of caffeine after a period of 1, 3 and 9 hours
10µM - 3h | CONTROL	
10µM - 9h | CONTROL	
3µM - 1h | 0µM - 1h	Dose-dependent effect of caffeine in gene expression of cells treated with **3 µM** of caffeine after a period of 1, 3 and 9 hours
3µM - 3h | 0µM - 3h	
3µM - 9h | 0µM - 9h	
10µM - 1h | 0µM - 1h	Dose-dependent effect of caffeine in gene expression of cells treated with **10 µM** of caffeine after a period of 1, 3 and 9 hours
10µM - 3h | 0µM - 3h	
10µM - 9h | 0µM - 9h	

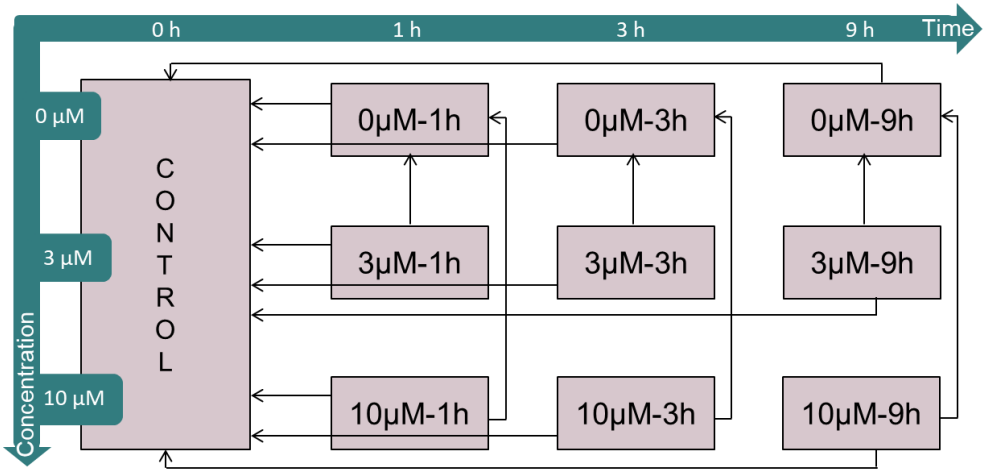

Figure 4.2 – Representation of all conditions sampled in this study and how they are compared. Arrows represent all combinations of conditions we compared in this study to identify genes responding to caffeine treatment. The results obtained from one comparison can be interpreted as the difference in gene expression of cells belonging to the condition in the beginning of the arrow comparing to cells belonging to condition in the end side of the arrow.

Gene Ontology and Pathway Analysis

Gene ontology was performed using ToppGene (*https://toppgene.cchmc.org*) with a FDR cutoff of 0.1 for biological process (BP) category. The input consists of a list of gene symbols of all genes represented by the TCs in study for each specific contrast described in the results section.

Pathway analysis was performed using WEBgestalt (*http://www.webgestalt.org/webgestalt_2013*), considering top 10 relevant pathways. The input consists of a list of gene symbols of all genes represented by the TCs in study for each specific condition described in the results section. A background list consisting of all genes represented in the data set was used to enrich the pathway analysis.

Enhancer Expression in Response to Caffeine Treatment

Differential expression for enhancers was performed in R with edgeR package 3.14.0 using the generalized linear model (GLM), the same method as the one applied to differential gene expression (Robinson et al., 2010). The same comparisons as in differential gene expression were applied in enhancer differential expression (Table 1; Figure 4.2). A cutoff of 0.1 FDR was used as significance level and no cutoff was applied for the fold change; meaning that any value indicating up- or downregulation was reported.

Enhancer-Promoter Expression Correlation

In order to associate enhancer expression with promoter, we first identified pairs of enhancers and promoters with the potential to be interacting. For this, we created an intersection table between the genomic localization of both of the intervenients and kept enhancer-promoter pairs that co-resided within 500,000 base pairs. The final list of candidate interactions accounted for 196,031 co-residing pairs. This list indicates how many possible pairs of enhancers and promoters are close enough in the genome to allow an interaction.

A correlation coefficient (Pearson correlation) was computed between enhancer CPM counts and promoter CPM counts, across 31 samples, for all the candidate pairs established from the previous intersection. P-value was adjusted to FDR and a cutoff of 0.1 for the latter was used to filter the table. To select the significant putative interacting pairs we applied a 0.5 threshold for the correlation coefficient. A total of 909 highly correlated enhancer-promoter pairs was obtained and further analyzed.

5. Results

The results are divided into two sections. Chapter 1, in which we address aim 1: identification of mediator genes in caffeine response. Chapter 2, in which we address aims 2 and 3: identification of active enhancers in neuronal cells and identification of enhancers regulating mediator genes in caffeine response. Before entering in the chapters mentioned above, we present a common section about the initial quality control performed on this data set, prior to the analysis reported in all chapters.

Data Quality, Outliers and Sample Consistency

After processing the sequencing results as briefly described before, we obtained a total of 43,631 tag clusters. Each of these has a corresponding number of read counts per sample, representing the expression detected. Given the dimension of the data set and the fact that a large fraction of the detected TCs show only basal expression levels, a filtering step was needed. 32 samples represent the 32 conditions tested in this study, each one consisting of a different combination of caffeine concentration and exposure time.

From the initial 43,631 TCs, 35,257 showed expression higher than one CPM in at least two samples and were kept for analysis. The 10 highest expressed TCs, also removed from analysis, represented housekeeping genes, miRNA and lncRNA genes, such as *tuba1*, *mir3917* and *malat1*, respectively. The final data set used for further analysis consists of 35,189 TSSs and was obtained after removal of 68 TCs with inconsistent expression levels between replicates.

From all 32 samples, sample C18 was shown to be an outlier. Visualization of expression levels distribution across all samples after filtering enlightens the fact that sample C18 shows a different profile from the rest of the samples. In Figure 5.1, we see the distribution of expression counts and sample C18 displays a shift in expression towards zero even after filtering of lowly expressed TCs (Figure 5.1A). This means that this particular sample has a high number of TCs with low or no expression detected. Unlike the homologous replicates for the same condition, C18 appears as an outlier. Normalization of the data for different library sizes does not improve the distribution of expression for sample C18 relatively to other samples, since this sample has a much smaller library size due to the high number or TCs with null expression. We concluded that this peculiar extent of null expression represented an imprecision in the library.

Principal component analysis was used as a method of dimensionality reduction and confirmed that sample C18 is, in fact, an outlier amongst all 32 samples (Figure 5.2). This sample is clearly separated from the rest of the samples by both principal components. PCA does not show overall differences between different conditions (treated and non-treated cells and different time points), as well as no batch effects. PCA plot after removing the outlier is shown in Figure 5.3A. Although we

expected to see clustering between replicates of the same group this is not the case, since the expression varies among replicates for the same condition. One reason can be the heterogeneity of the population, which is estimated as 80-90% of neurons and 10-20% of glial cells. The stage of differentiation can also influence the results, since we believe that the population is not completely homogenous regarding the differentiation stage, although we estimate that the majority of the population represents completely/fully differentiated cells, mainly mature neurons. PCA plot does not show clustering of samples based on time or concentration points (Figure 5.3, B and C, respectively). Final data set consists of 35,189 TCs and 31 samples.

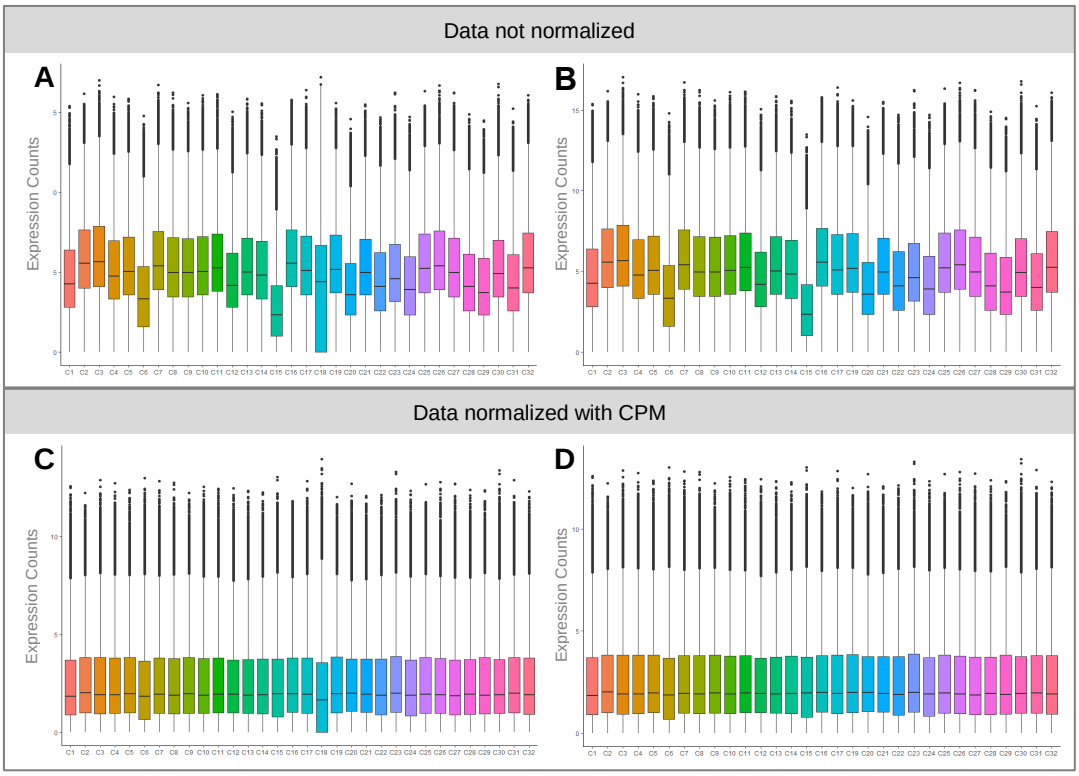

Figure 5.1 – Distribution of, normalized and not normalized, tag cluster expression counts of filtered data before and after removing outlier sample. Box plots for n=35,189 representing the distribution of individual expression counts detected for each of the filtered tag clusters before and after CPM normalization (upper and lower panel, respectively). **A)** Not-normalized data before removing sample C18; **B)** Not-normalized data after removing sample C18; **C)** Normalized data before removing sample C18; and **D)** Normalized data after removing sample C18. Box plots are colored according to sample name; median (solid horizontal line), upper and lower quartiles (upper and lower limits of box, respectively) are represented.

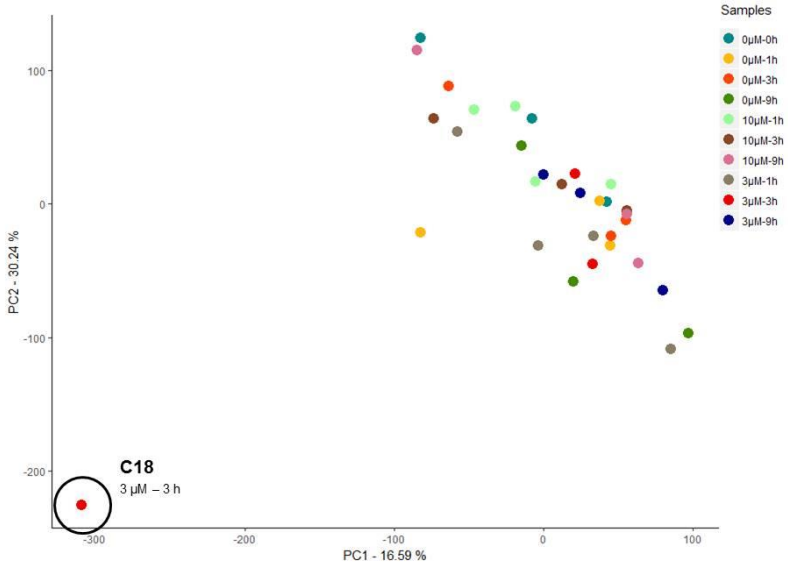

Figure 5.2 – Overall differences in gene expression of all samples for normalized and filtered data. Principal component (PC) analysis plot illustrating the differences in normalized expression counts of high-dimensional data consisting of 35,189 tag clusters. Replicates are colored according to experimental condition. No visible clusters or batch effects are identified. One outlier sample is identified (C18), clearly separated from the rest of the samples along PC1 and PC2.

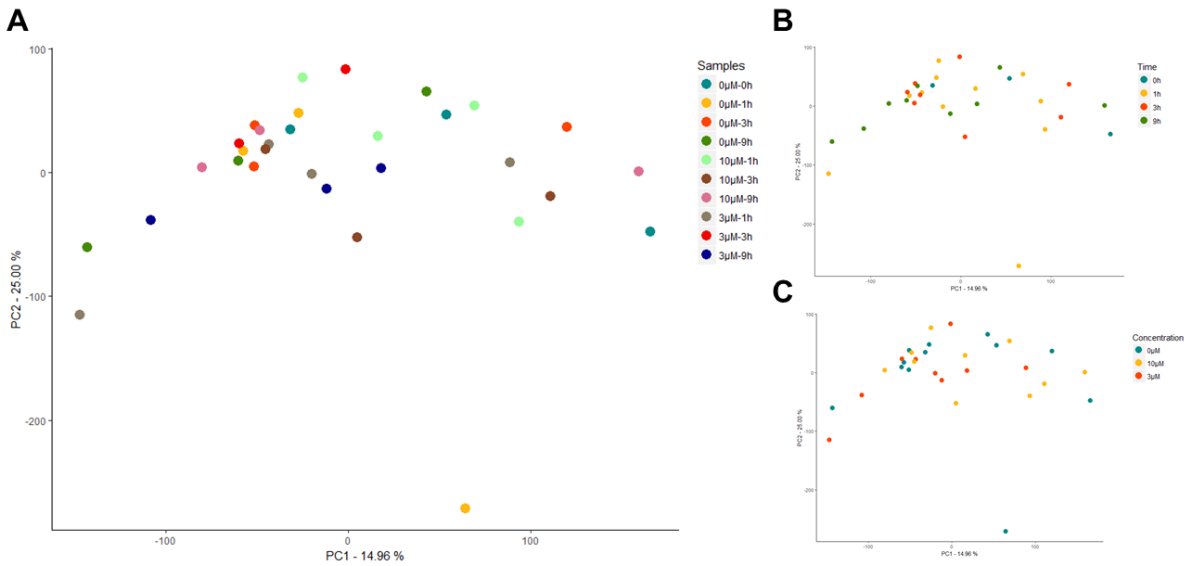

Figure 5.3 – Overall differences in gene expression of all samples for normalized and filtered data. Principal component (PC) analysis plot illustrating the differences in normalized expression counts of high-dimensional data consisting of 35,189 tag clusters. Replicates are colored according to experimental condition **(A)**, time **(B)** and concentration **(C)**. No visible clusters or batch effects are identified.

CHAPTER 1: How does gene expression mediates caffeine response of neuronal cells and what genes mediate this response?

Gene Expression in Neuronal Cells

A total of 43,631 tag clusters were detected in the sequenced CAGE libraries. Each tag cluster represents a group of TSSs that is associated with a gene. Several TSSs are annotated with the same gene, which indicates that one gene can have multiple transcription start sites, or alternative promoters. From all TCs detected, 6,062 are not annotated and the remaining represents 14,014 genes.

For all the comparisons presented in Table 5.1, a total of 307 differentially expressed tag clusters was obtained. From all differentially expressed genes, only 268 are annotated and represent 253 genes. In Supplementary Table 1, the list of differentially expressed tag clusters and corresponding genes can be found, including information on the comparisons used, fold change and statistical significance.

Gene Expression Changes Due to Handling

When analyzing non-treated cells over time, we observed accentuated changes in gene expression: 15, 44 and 38 annotated TCs were up- or downregulated after 1, 3 and 9 hours, respectively (Figure 5.4, yellow bars). The changes seen for these transcription start sites do not represent a response to caffeine, since these cells were not submitted to caffeine treatment. These alterations might represent the result of sample handling and cell vital processes that are active in these cells. These variations, considered as background variation, are much accentuated and can significantly mask the response to caffeine stimulus in treated cells if not considered.

The genes represented by the mentioned TCs with altered expression are involved in processes such as regulation of transcription from RNA polymerase II promoter (GO:0006357), regulation of cell differentiation (GO:0045595), positive regulation of cellular biosynthetic process (GO:0031328), positive regulation of signaling (GO:0023056), regulation of multicellular organismal development (GO:2000026), neuron differentiation (GO:0030182), positive regulation of phosphorylation (GO:0042327), cell motility (GO:0048870), tissue morphogenesis (GO:0048729), regulation of MAP kinase activity (GO:0043405), and cell migration (GO:0016477). These GO terms were obtained as described in section Methods.

Table 5.1 – Number of differentially expressed tag clusters. List of all contrasts used in differential expression analysis and the correspondent total number of differentially expressed tag clusters, annotated tag clusters and the genes correspondent to each annotated tag cluster.

Comparison	Tag clusters	Annotated tag clusters	Genes
0µM - 1h | CONTROL	19	15	13
0µM - 3h | CONTROL	48	44	42
0µM - 9h | CONTROL	46	38	38
3µM - 1h | CONTROL	26	23	19
3µM - 3h | CONTROL	13	12	12
3µM - 9h | CONTROL	2	2	2
10µM - 1h | CONTROL	24	21	17
10µM - 3h | CONTROL	43	37	36
10µM - 9h | CONTROL	1	-	-
3µM - 1h | 0µM - 1h	-	-	-
3µM - 1h | 0µM - 1h	1	1	1
3µM - 9h | 0µM - 9h	-	-	-
10µM - 1h | 0µM - 1h	-	-	-
10µM - 3h | 0µM - 3h	84	75	73
10µM - 9h | 0µM - 9h	-	-	-

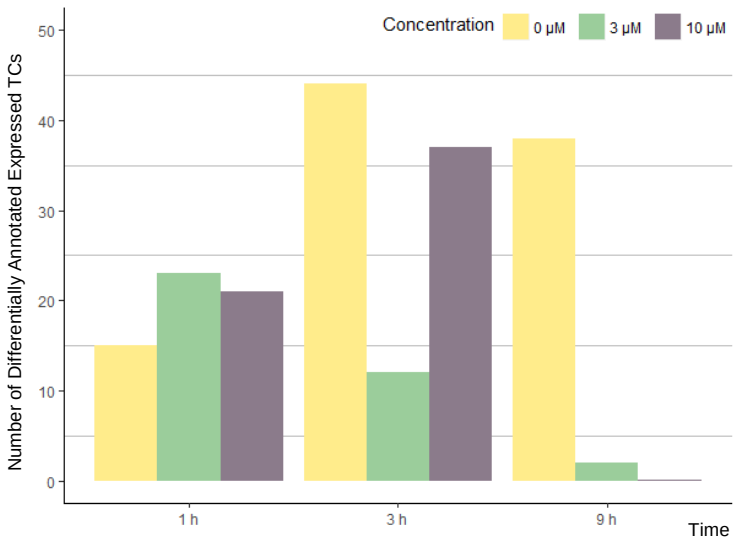

Figure 5.4 – Gene expression changes regarding non-treated cells and cells treated with 3 and 10 µM of caffeine in comparison with control samples. For all three different concentrations (yellow, green and purple bars), the number of differentially expressed tag clusters is displayed for each time point of RNA collection. The number of differentially expressed TCs results from a comparison between all indicated conditions and the control sample in the beginning of the experiment. Only annotated TCs are considered. After 9 hours, caffeine does not induce significant changes in gene expression; however, non-treated cells still present a considerable extent of alterations in gene expression.

Gene Expression Changes in Cells Treated with Caffeine

Differential expression analysis allows an evaluation of differences in gene expression in different conditions. With our first question in mind, it allows assessing how different concentrations of caffeine affect gene expression during the course of time. If a specific gene is differentially expressed between two conditions, then caffeine is causing a change in its expression level.

In this study, we are particularly interested in gene expression changes that occur due to caffeine exposure, thus we compare non-treated cells with cells treated with 3 μM and 10 μM at the same time points to see the effect of concentration. We also compare control cells (no treatment at 0 h) with 3 μM and 10 μM treated cells at all different time points to see the effect over time.

A wide number of different comparisons can be made in order to investigate what is the effect over time, the concentration effect, which parameter (time/concentration) has the bigger impact on gene expression variation, amongst other. However, it is not feasible to address all at once. With this in mind, and focusing on our first aim, we evaluated how cells respond to different concentrations of caffeine in the different time points, removing the background noise that is observed in non-treated cells.

In respect to treated cells, the comparisons made to assess the time-dependent response to caffeine resulted in 109 differentially expressed tag clusters, from which 95 are annotated (Figure 5.4, green and purple bars) and represent 86 genes. The comparisons made to assess the dose-dependent response to caffeine resulted in 85 differentially expressed tag clusters, from which 76 are annotated and represent 74 genes. Non annotated tag clusters were considered in later correlation analysis with enhancer expression but were omitted in gene ontology and pathway analysis.

Principal component analysis was, once again, used to visualize and summarize the high dimension transcriptional data to identify caffeine-induced differences in the gene expression patterns of cells (Figure 5.5). When considering all genes that exhibit gene expression changes in response to caffeine, samples appear to group according to time of exposure. Samples corresponding to 1 hour of exposure are very different, in their patterns of gene expression, from samples corresponding to 9 hours of exposure, regardless of the caffeine dose. The 9 hour samples are very similar to control samples, which might indicate that gene expression returns to normal levels after 9 hours of caffeine treatment.

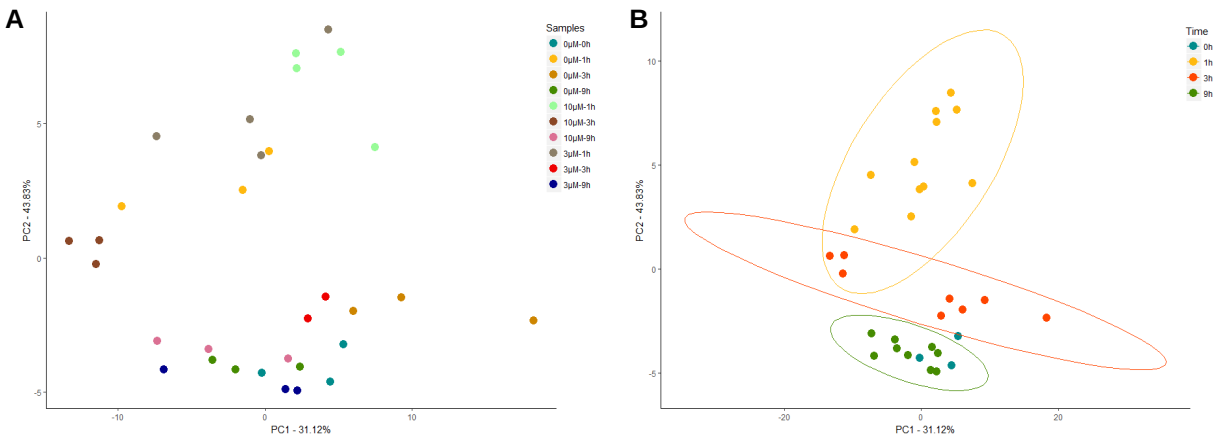

Figure 5.5 – Caffeine-induced gene expression differences. Principal component (PC) analysis plot illustrating the differences in normalized expression counts of high-dimensional data consisting of 197 tag clusters, corresponding to all tag clusters responding to caffeine treatment (3 and 10 µM). Replicates are colored according to experimental condition **(A)** and time **(B)**. Ellipses represent 95% confidence intervals. **A)** Differences between samples are more evident due to caffeine induction of expression of different sets of genes according to treatment. **B)** PC2 separates samples in 3 individualized clusters. Clustering of control samples and 9 hour samples suggests that gene expression patterns of these samples are similar.

Genes Mediating Response to Caffeine Stimulus

The changes in gene expression that are observed in non-treated cells are expected to occur in treated cells as well. These alterations are probably a result of sample manipulation, such as removing cells from incubation every time a measurement needs to be made. In order to assess the effect of caffeine alone, we need to be able to distinguish the changes that occur due to handling from the changes that occur in response to caffeine addition to the medium.

Removal of background noise was possible by integrating differential gene expression results from three comparisons: 1) compare each time point for 3 µM and 10 µM with control; 2) compare the time point for non-treated cells with control; and 3) compare the same time point in treated and non-treated cells. As an example, evaluation of caffeine response in cells treated with 3 µM after 3 hours is represented by the following comparisons: 1) 3µM-3h|CONTROL; 2) 0µM-3h|CONTROL; and 3) 3µM-3h|0µM-3h, where “CONTROL” represents 0µM-0h (Figure 4.2).

By comparing cells treated with caffeine at different time points with the control cells we look at the effect of caffeine over time for each concentration. The same comparison can be made for non-treated cells, and in this case we mainly see the results of sample manipulation. The simple act of removing cells from incubation several times during the experiment has effects on gene expression as cells struggle to efficiently respond and compensate even the slightest stress that might be triggered in the cells. When considering these two comparisons we can see the common genes changes and investigate if these changes are completely due to handling or if caffeine might have a role in amplifying the alterations. We address this question by comparing the fold change in both conditions

and selecting the genes that show a significant difference. Last, we compare our condition of interest with non-treated cells at the same time point to see the effect of concentration only, regardless of the evolution of gene expression over time.

After comparing each concentration at each time point with the correspondent condition for non-treated cells, we observe significant changes in gene expression at 1 h for cells treated with 3 µM of caffeine and at 3 h for cells treated with 10 µM of caffeine. No other dose-dependent differentially expressed genes were found for time points other than the ones stated.

By means of Venn diagrams illustrations, the relation between all three comparisons can be studied as a whole and a final list of genes can be selected. The Venn diagram shown in the left panel of Figure 5.6 illustrates the number of differentially expressed genes observed at 1 h for cells treated with 3 µM of caffeine when compared to the control (A) and when compared to non-treated cells at the same time point (B) as well as the number of differentially expressed genes due to handling (C). In the right panel of the same figure, a schematic of the comparisons made is shown to support the interpretation of the venn diagram.

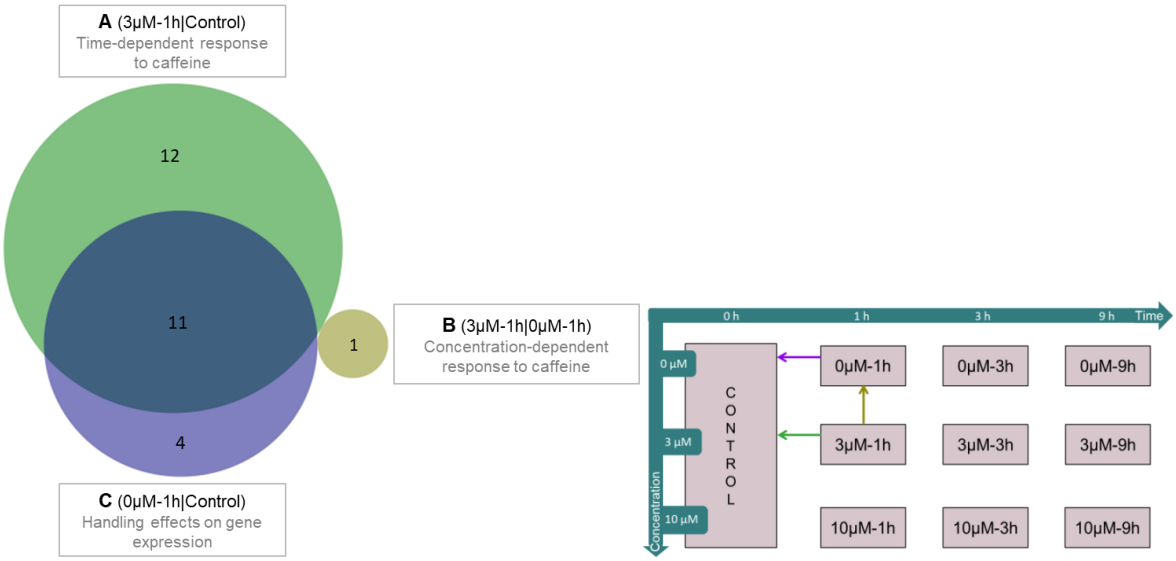

Figure 5.6 – Evaluation of gene expression responses to treatment with 3 µM of caffeine. On the left side, a venn diagram represents the number of differentially expressed genes in each comparison: **A)** time-dependent response, **B)** concentration-dependent response and **C)** response to sample handling. The number of differentially expressed genes that are common in non-treated cells and in cells treated with 3 µM of caffeine is also shown. On the right side, a schematic of the comparison used in the analysis with colored arrows indicating the comparisons shown in the venn diagram representation.

After removing the genes expressed as a result of manipulation, the resulting list of 16 genes was further investigated to assess the nature of these genes as well as the pathways and processes they relate to.

Gene ontology analysis (Figure 5.7) showed a total of 61 GO terms within the established parameters, with 22 of which hereby reported as the most worth highlighting. Six GO terms related to synaptic activity processes are perturbed, such as *synaptic signaling* (GO:0099536), *trans-synaptic signaling* (GO:0099537), *anterograde trans-synaptic signaling* (GO:0098916), *chemical synaptic transmission* (GO:0007268), *positive regulation of dopamine uptake involved in synaptic transmission* (GO:0051586) and *positive regulation of neurotransmitter uptake* (GO:0051582).

One of 7 down-regulated genes is related to the two latter processes: *nat8l*, a gene coding for a neuron-specific metabolite – N-acetylaspartate acid (NAA) – that, ultimately, regulates acetyl co-A transport to nervous system and promotes dopamine uptake (Arun *et al*., 2009; Moffet *et al*., 2013). When the process is down-regulated, acetyl co-A supply to the nervous system is strongly conditioned, which happens after 1 hour of caffeine exposure. *Nat8l* is also involved in all 4 synapse-related processes earlier mentioned, as well as three up-regulated genes: *egr2*, *egr3* and *npas4*. Early growth response (EGR) genes strongly condition neuronal plasticity and are, often, involved in memory studies (Minatohara *et al.*, 2015).

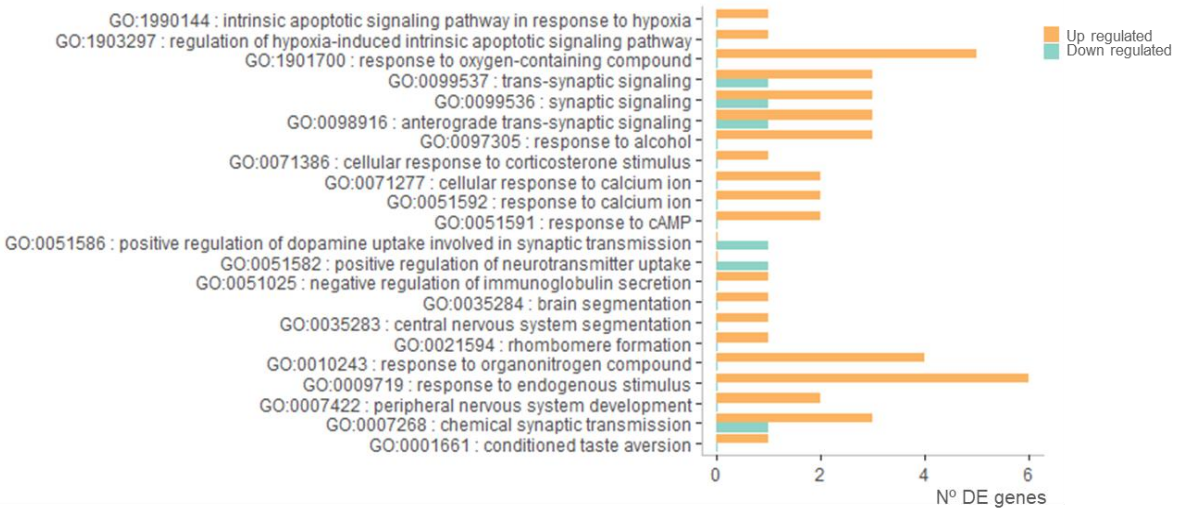

Figure 5.7 – Biological processes perturbed by treatment with 3 µM of caffeine. Gene ontology (GO) analysis results performed in order to understand in which biological processes the caffeine response mediator genes are involved. P-values are indicated in Supplementary Table 3.

Several pathways appear to be perturbed at 1 hour after exposing cells to 3 µM of caffeine (Figure 5.8; Supplementary Figures S1-S3). With a an increase in expression of 16x, *Fos* is involved in B and T cell receptor signaling pathways and toll-like receptor signaling pathways, which suggests an upregulation of immune response processes. One significantly downregulated gene – *TubaA* – is associated with gap junction establishment, with a decrease in expression of around 15x.

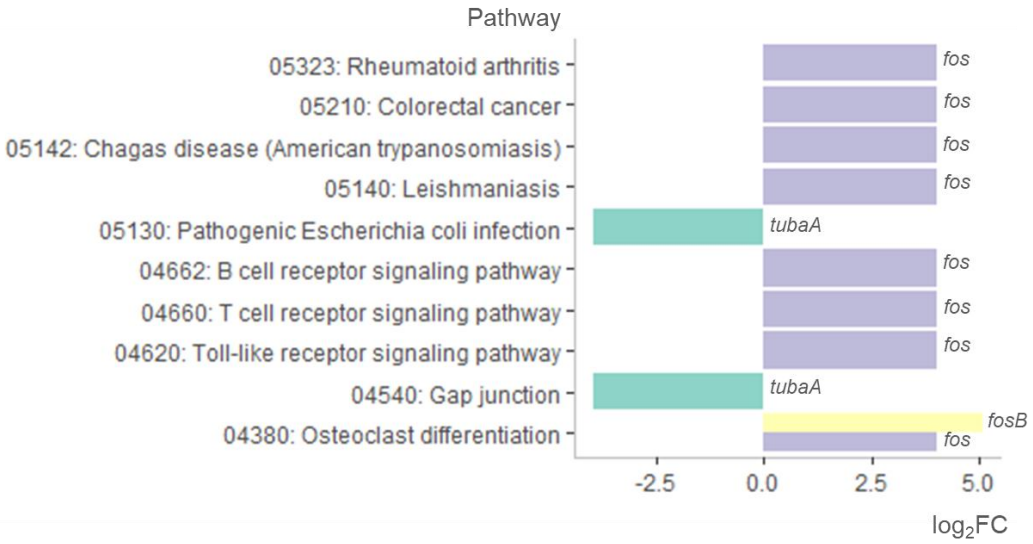

Figure 5.8 – Biological pathways perturbed by treatment with 3 µM of caffeine. Pathway analysis results performed in order to understand in which biological pathways the caffeine response mediator genes are involved.

A similar diagram is shown for cells after 3 hours of exposure to 10 µM of caffeine (Figure 5.9), illustrating the number of differentially expressed genes observed for these cells when compared to the control (A), when compared to non-treated cells at the same time point (B) as well as the number of differentially expressed genes due to handling (C). Once again, in the right panel of the figure, a schematic of the comparisons made is shown to support interpretation. Genes expressed as a result of manipulation were removed and a final list of 96 TCs, was further investigated with pathway and gene ontology analysis.

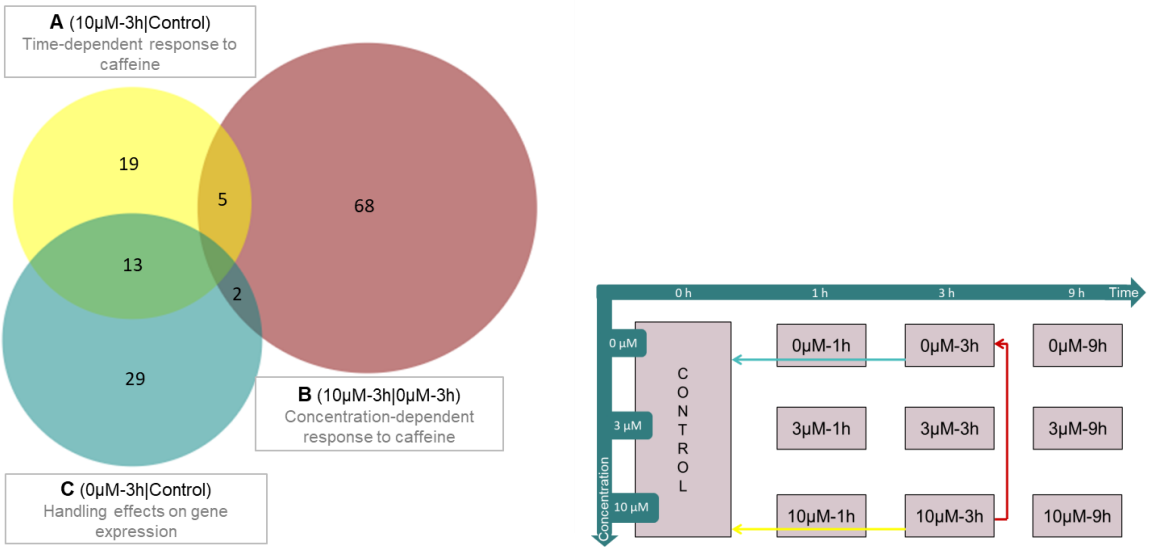

Figure 5.9 – Evaluation of gene expression responses to treatment with 10 µM of caffeine. On the left side, a venn diagram represents the number of differentially expressed genes in each comparison: **A)** time-dependent response, **B)** concentration-dependent response and **C)** response to sample handling. The number of differentially expressed genes that are common in non-treated cells and in cells treated with 10 µM of caffeine is also shown. On the right side, a schematic of the comparison used in the analysis with colored arrows indicating the comparisons shown in the Venn diagram representation.

Gene ontology analysis (Figure 5.10) showed a total of 14 GO terms with FDR<0.1; the number of genes associated with each term is indicated in the figure. Regulation of gap junction assembly (GO: 1903596), cell projection organization (GO: 0030030), neuron differentiation (GO: 0030182), regulation of synapse structure or activity (GO: 0050803), regulation of synaptic plasticity (GO: 0048167), neurogenesis (GO: 0022008) and positive regulation of cell communication (GO: 0010647). Overall, processes related to cell projection organization and generation of neurons appeared to be decreased in cells after a period of 3 hours of exposure to 10 µM of caffeine.

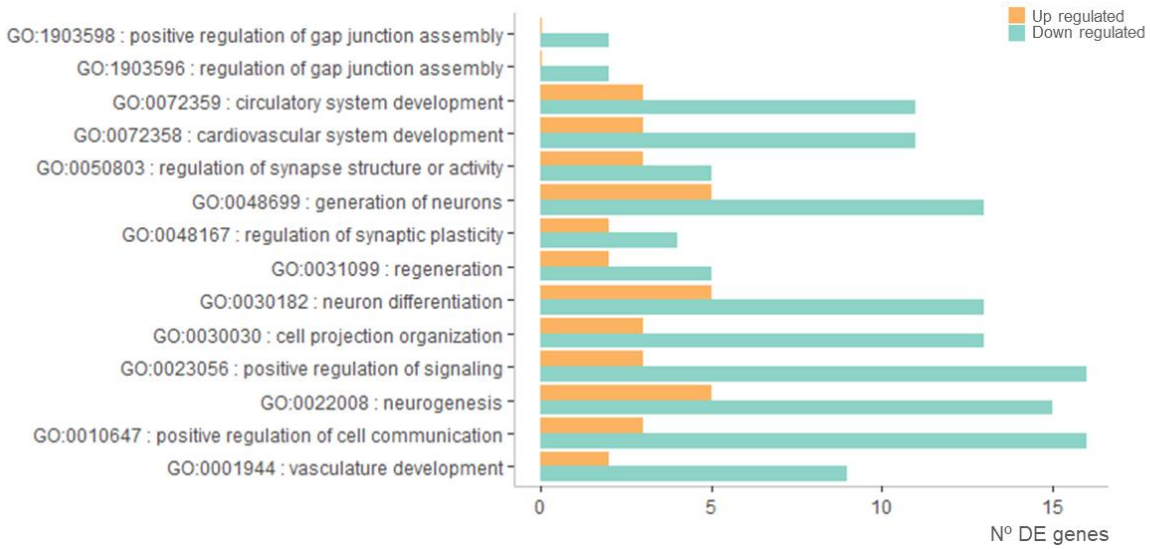

Figure 5.10 – Biological processes perturbed by treatment with 10 µM of caffeine. Gene ontology (GO) analysis results performed in order to understand in which biological processes the caffeine response mediator genes are involved. P-values are indicated in Supplementary Table 2.

Regarding the pathway analysis (Figure 5.11), a total of 10 pathways appear to be perturbed by 10 µM of caffeine after 3 hours of addition. Two up-regulated genes are part of *cell adhesion molecules* (ID: 04514) pathway. More precisely these genes are part of the cell adhesion molecules in the nervous system, mediating close proximity interactions between pre and pos-synaptic neurons as well as glial cells and neurons. The genes, *Nrxn3* (neurexin 3) and C*ntn1* (contactin 1) present a fold change in expression of 2 and 2.5, respectively, greatly contributing for an overall up-regulation of neuronal communication processes. Other significant pathway altered in this condition is *complement and coagulation cascades* (ID: 04610), which presents 3 down-regulated genes: *C5ar1* (complement component 5a receptor 1), *Plat* (plasminogen activator, tissue-type) and *a2m* (alpha-2 macroglobulin). *C5ar1* is a mediator of the complement system and it is known to promote degranulation and chemotaxis, essential processes for inflammation and immune response. Its down-regulation in neuronal cells suggests an important neuroprotective role in the brain. *A2m* can disrupt coagulation processes and is known to be implicated in Alzheimer's disease, as it mediated the clearance and degradation of A-beta. *Plat*, a down-regulated gene involved in the coagulation cascade, appears to decrease this process in cells exposed to caffeine. Axon guidance pathway appears to be decreased

as well as neuroactive ligand-receptor interaction. Pathways are shown in Supplementary Figures S4-S7.

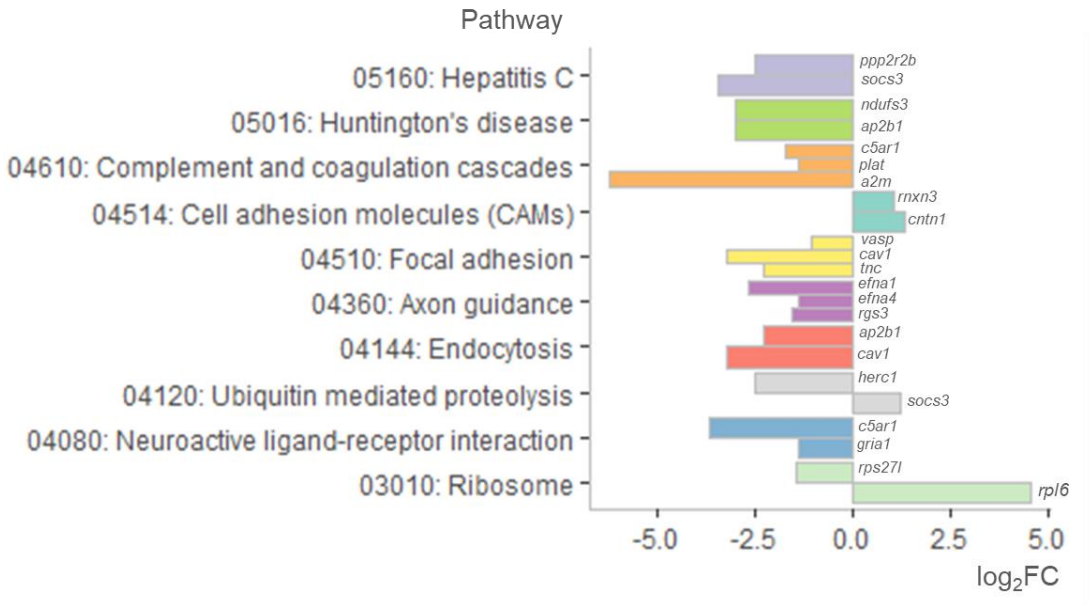

Figure 5.11 – Biological pathways perturbed by treatment with 10 µM of caffeine. Pathway analysis results performed in order to understand in which biological pathways the caffeine response mediator genes are involved.

CHAPTER 2: Identification of active enhancers in neuronal cells and identification of enhancers that regulate mediator genes in caffeine response

Enhancers in Neuronal Cells

The genomic localization of active transcribed enhancers was predicted as described and 8,061 enhancers were identified by the predictor. The 15 highest expressed enhancers were visually investigated in depth in terms of shape, expression and genomic localization, using ZENBU genome browser based on human genome 19 (hg19) for reference.

The 15 highest expressed enhancers showed bimodal and bi-directional patterns of expression. The CAGE peaks and the enhancer representation are displayed in Figure 5.12, which illustrates the bi-directionality of typical enhancer expression. Enhancers overlapping with coding genes were investigated regarding the genome localization and were found to be located within intronic regions. One enhancer was found to overlap with two TCs of annotated genes, even though the predictor excluded these cases. Since one of the TCs is annotated as long non-coding RNA and its function is not known so far, it can be the case that this is a real enhancer.

Enhancer Activity in Response to Caffeine Stimulus

A total of 12 enhancers were found to be differentially expressed between different conditions, 7 of which overlap with coding genes and were confirmed to be in intronic regions (Table 5.2). Comparisons between consecutive time points for each concentration were included in the analysis and 1 enhancer (chr17:42589128-42589447) is differentially expressed throughout time in cells treated with 3 µM of caffeine. The expression of this enhancer increases between hour 1 and hour 3 and decreases until hour 9 (Figure 5.13).

Table 5.2 – Number of differentially expressed enhancers. List of all contrasts used in differential expression analysis that showed differentially expressed enhancers and the correspondent number of differentially expressed enhancers.

Comparison	Enhancers
0µM - 3h | 0µM - 1h	3
3µM - 1h | CONTROL	8
3µM - 9h | CONTROL	1
3µM - 3h | 3µM - 1h	1
3µM - 9h | 3µM - 3h	1

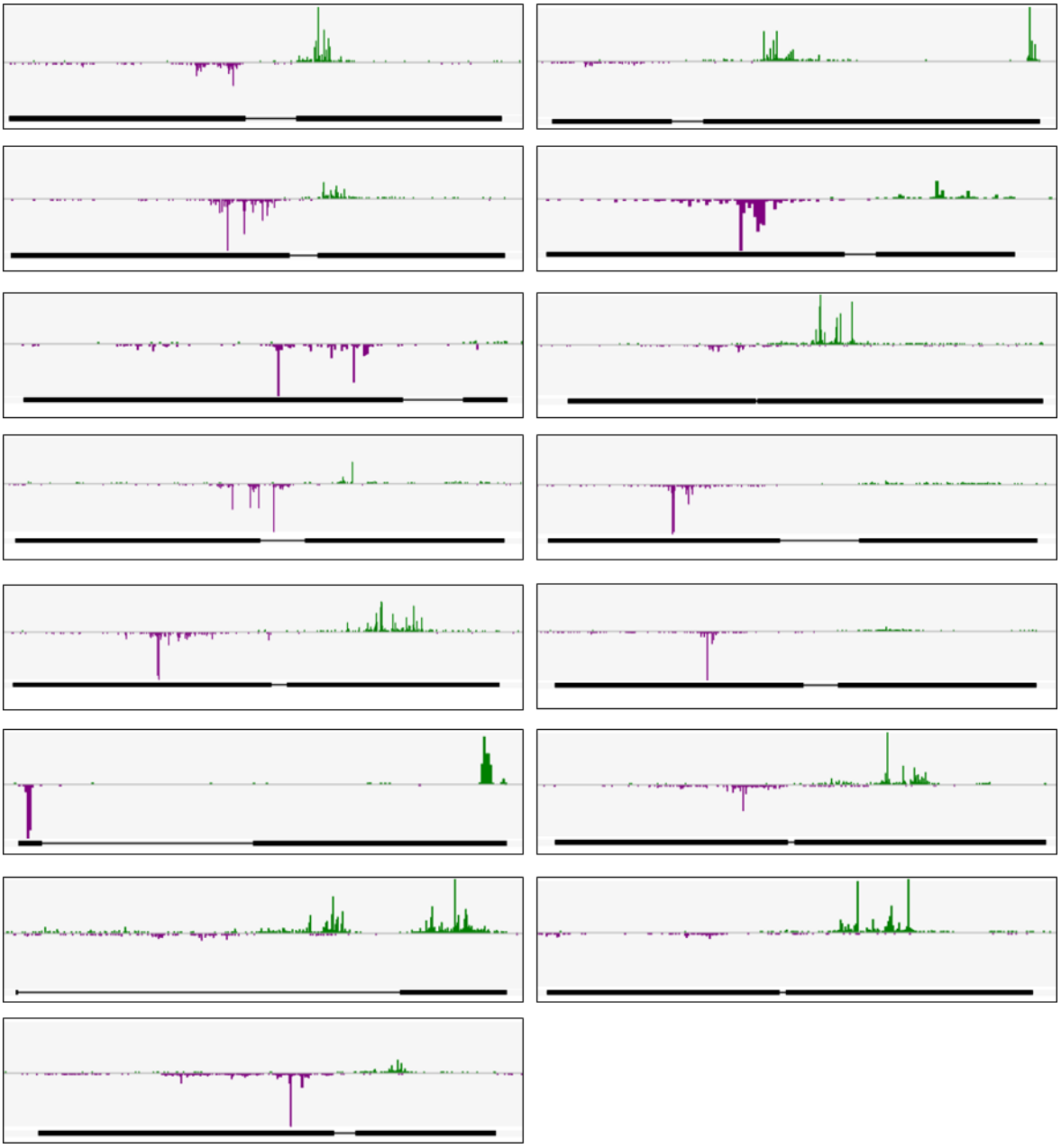

Figure 5.12 – Representation of bimodal and bidirectional expression pattern of 15 highest expressed enhancers in neuronal cells. Green peaks represent expression detected on the forward strand and purple peaks represent expression detected on the reverse strand. Black boxes connected by horizontal line indicate the enhancer region.

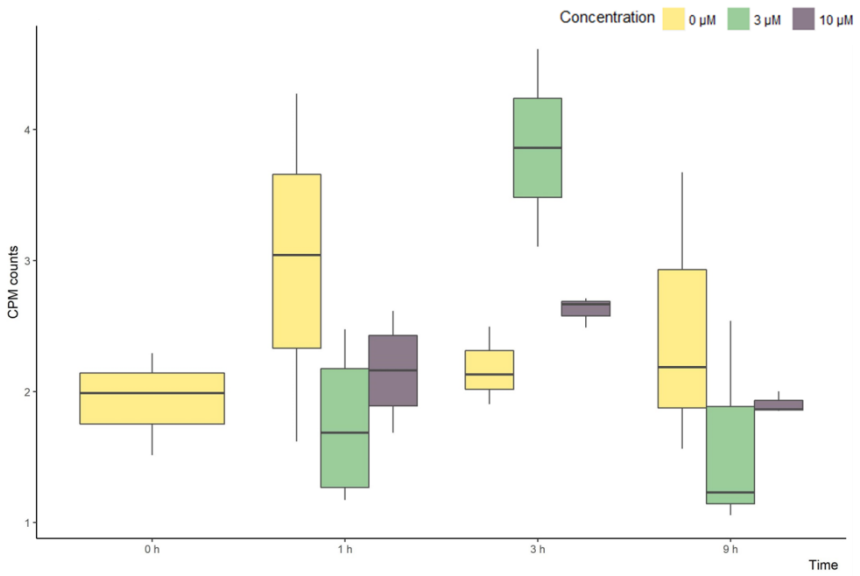

Figure 5.13 – Evolution of enhancer expression over time. Enhancer chr17:42589128-42589447 shows a clear response to treatment with 3 and 10 µM of caffeine (green and purple bars). After 3 hours of exposure, 3 µM-treatment appears to have a strong effect on the induction of this enhancer.

Caffeine Mediator Genes Regulated by Enhancers

A pairwise correlation in expression levels between enhancers and promoter regions was performed for all intersection pairs and we identified 909 intra chromosomal pairs with high correlation coefficient (cor>0.5), with an average of 1.2 strongly associated TCs per enhancer.

In the same fashion as before, the 15 highest expressed enhancers were assessed in depth and from all the promoter-enhancer pairwise combinations regarding these 15 enhancers, we identified 52 intra chromosomal pairs with a high correlation coefficient (cor>0.5). We further investigated what was the nature of the genes represented by TCs in close interaction with these highly expressed enhancers. Genes involved in cell cycle regulation and cell proliferation (*Malat1*), centrosome cohesion before cell division (C*rocc*), neuroprotective and anti-apoptotic factor (*Mtrnr2l1*), cell signal transduction (C*dc37*), nervous system development (*GamT*), cytoskeleton regulation in neurons and cell-cell adhesion A*pc2*), intracellular protein transport (*Reep6*), among several other.

We also looked for enhancers that could potentially be regulating the differentially expressed TCs. Nine differentially expressed TCs have a strong association with one enhancer. Even though the target TC was differentially expressed, none of the associated enhancers were differentially expressed in the same contrast. Concerning differentially expressed enhancers, only 1 shows a strong association with two promoters.

Considering the genomic localization of the 63 identified enhancer-promoter pairs that show strong correlation in expression levels, 8 enhancers were found to be co-localized with the promoters. This means that the expression detected for the enhancer in one direction corresponds to the tag cluster representing the promoter, which explains the high correlation coefficient. For all cases, the tag clusters are not annotated. Since the genes are not known, the tag clusters might, in fact, constitute an enhancer. Here, we report some of the enhancer-promoter pairs with good correlation in expression that look relevant and worth investigating (Figure 5.14).

C5ar1, a significantly differentially expressed gene is associated with enhancer chr19:47949383-47949406 with a correlation coefficient of 0.73 (Figure 5.14, A). Unlike the target gene, the enhancer is not differentially expressed in any condition. The expression of the TC is higher at hour 3 for non-treated cells. When cells are treated with 3 µM of caffeine, expression of the enhancer appears to be downregulated and, consequently, so is the expression of the promoter. With 10 µM treatment, the enhancer is not expressed at all and the expression of the promotor strongly decreases. Although, these changes are not noticeable if comparing treated cells with control cells, the results suggest that the enhancer is regulating this gene and that the gene is a mediator of caffeine response. Caffeine appears to contradict the upregulation of C*5ar1* expression in non-treated cells.

Sfxn3, a gene that presents changes in gene expression in non-treated cells after 9 hours, appears to be regulated by enhancer chr10:103064180-103064576. However, the expression of the promoter of this gene seems to change in the opposite way of the enhancer. In Figure 5.14B, the expression of the enhancer is gradually increasing in non-treated cells as opposed to the expression of the promoter, which is progressively decreasing. This might suggest that this enhancer regulates an intermediary gene that in turn regulates *Sfxn3*.

The promoter of *Nfix* is downregulated at 3 hours in cells treated with 10 µM of caffeine. When evaluating the relation between the expression of the promoter and the expression of the enhancer, it is clear that the absence of enhancer expression is mirrored is the strong downregulation seen in the promoter (Figure 5.14C).

A high correlation in expression between a promoter and the associated enhancer is seen with the promoter of *Sox2*, a transcription factor with a key role in maintenance of pluripotency state (Figure 5.14D). In all conditions, the expression of the promoter appears to follow the one of the enhancer. Another example of very high correlation in expression patterns is the promoter of *Dlgap1-as1*. Cells treated with 10 µM of caffeine appear to have a strong decrease in enhancer expression after 3 hours of exposure and, consequently, the same decline is seen in the evolution of the promoter expression.

One enhancer (chr17:42589128-42589447, Figure 5.13) is associated with two tag clusters (chr17:42429502..42429563,+ and chr17:42589267..42589277,-). The first one represents the promoter of *Grn*, a gene involved in inflammation, wound repair, and tissue remodeling processes.

The second one is not annotated and, thus, its function is unknown. This enhancer appears to be responding to treatment with 3 µM caffeine, since it shows significant upregulation after 3 hours followed by an accentuated downregulation until 9 hours. The second promotor co-localizes with the enhancer. As mentioned before, this might suggest the genomic region identified as a transcription start site of an non-annotated gene might, in fact, be an enhancer region that regulates the promoter of *Grn*.

A very highly expressed enhancer (chr7:157483026-157484172) shows a very high correlation (0.98) with *Ptprn2* promoter expression. However, the enhancer is located in an intronic region of this gene. Given that the enhancer is located more than 800 kbp away from the start of the gene, which is over 1 million bp in size, it might be a real intronic enhancer.

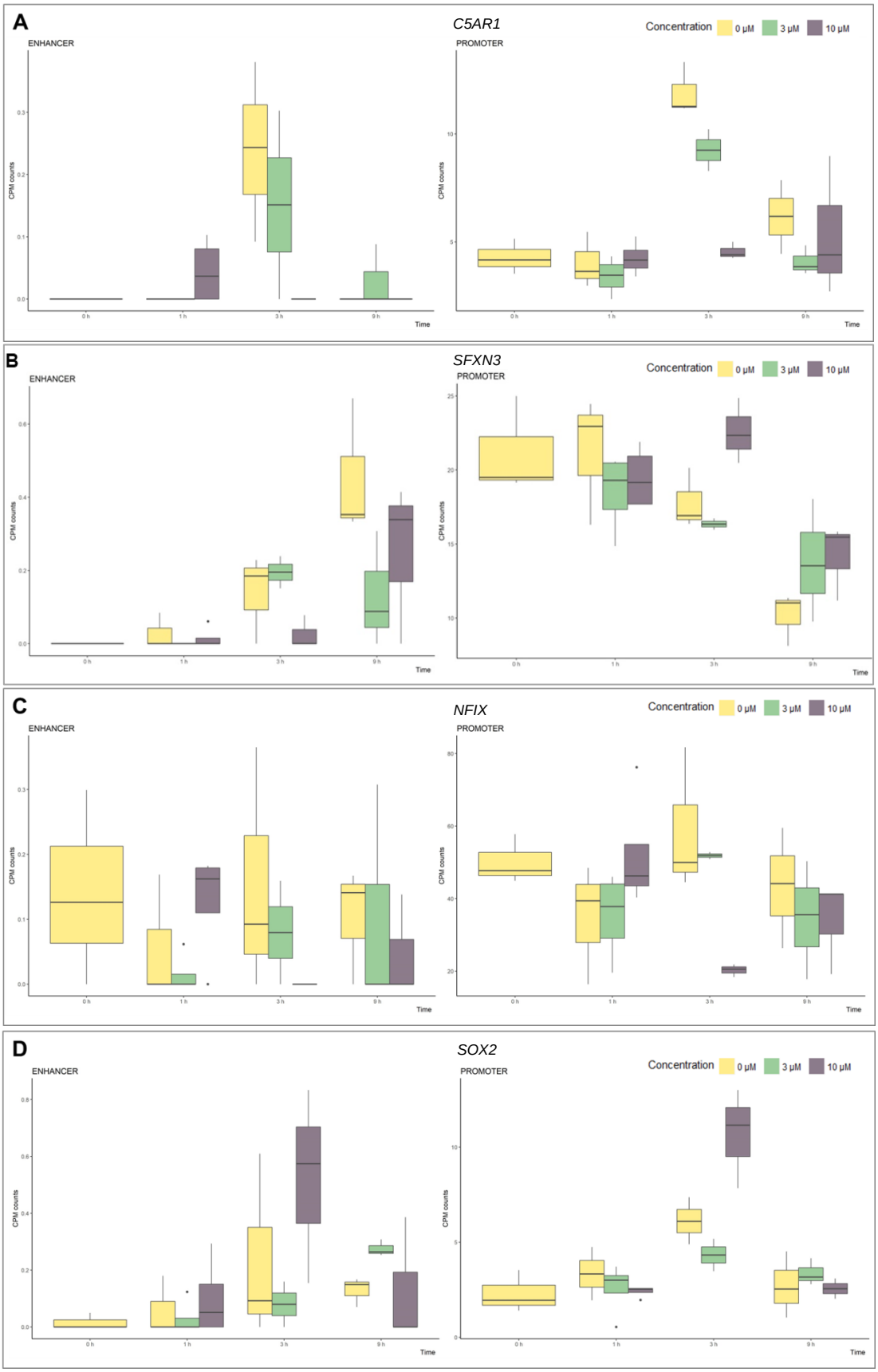

Figure 5.14 – Evolution of enhancer and promoter expression over time of putative interactions. On the left side, the enhancer expression in CPM-normalized counts. On the right side, the promoter expression in CPM-normalized counts. All pairs represent enhancer-promoter pairs with high correlation in expression, regarding time and caffeine treatment.

6. Discussion

The design of this study offers several advantages over other models used in similar studies. Studies regarding neuronal cells have been performed in animal models or neuroblastoma cell lines (SH-SY5Y), which are already very well characterized and implemented in research. The latter shows specific adrenergic and dopaminergic phenotypes, a feature required for disease-modeling studies, for instance. However, differentiation of iPSCs can be directed towards specific neuronal phenotypes and obtain enriched cell lines. A more general cell population, such as the one obtained in this study, is a good model for nervous system development or neurotoxicity studies due to its heterogeneity in cell types.

In a study concerning the effects of caffeine on the human brain, and being impossible to use a human brain as a model, a healthy human cell line appears very appealing and a major advantage over commonly established models. The use of human neuronal cells derived from human iPSCs resembles a healthy human nervous system cell population and stands as one of the major advantages of this study. Detection of gene expression with a high throughput genome-wide approach such as CAGE offers great coverage for the questions we proposed to answer in the present study. Other commonly used RNA-sequencing techniques detect expression on transcript level. CAGE allows the identification of promoter expression with high sensitivity and specificity, as well as non-annotated transcriptional enhancers (Shiraki *et al*., 2003).

Most studies resort to high concentrations of caffeine in the uncertainty that low concentrations would induce any kind of response by the cells or to try to attain similar results of those of long-term effects. Low concentrations of caffeine have been shown to induce antagonism of adenosine receptors. Here we show that low concentrations of caffeine have effects on specific genes' expression, as well as on regulator enhancers.

In chapter 1, we address our first aim of understanding what genes are responsible for mediating caffeine response in neuronal cells. For this we sampled cells that underwent treatment with different physiological concentrations of caffeine for 3 different exposure periods. A negative control was also sampled and consists of cells that were not exposed to caffeine and that were sampled in hour 0 of the experiment. To increase the precision in measuring gene expression changes, these cells were also sampled in all time points of the experimental design (1, 3 and 9 hours). With these 4 controls we can follow gene expression variation on non-stimulated conditions (no caffeine stimulus), considering that manipulation and sample handling interfere with gene expression.

To identify the genes responding to caffeine and to measure this response in terms of fold change, we used differential gene expression tools. To detect changes in gene expression we always compare two conditions and, for each gene, we obtain the difference between the expression of that gene in

both conditions in terms of fold change, always considering the statistical significance of that fold change.

The variations found in the transcriptome of non-treated cells are essential to understand and distinguish caffeine response and manipulation effects on cells. First of all, these cells show prominent activity in biosynthetic processes, cell motility and migration, tissue morphogenesis, phosphorylation and cell signaling which are vital and natural occurring processes in living cells. It is also expected to see up- and downregulation of these processes over time, which is the case. Genes involved in cell differentiation processes also show prominent changes over time which is indicative of the existence of a still-differentiating fraction of the cell population. We also detected up- and downregulation in the expression of genes involved in neuron differentiation processes over time. Upregulation of these genes shows that throughout 9 hours some cells are still differentiating and downregulation of the same genes suggests that a fraction of the cell population is undergoing differentiation and that this process is finalized during the experiment.

Three important points concerning treated cells are worth emphasizing. First, evaluating the evolution of gene expression by comparing all conditions with the control sample (no treatment at hour 0) illustrates a time-dependent response to caffeine. For a specific dose, we see the change throughout time. Secondly, evaluating gene expression changes by comparing all conditions with non-treated cells at 1, 3 and 9 hours illustrates a dose-dependent response to caffeine. For each specific time point, we see the difference between the expression profile of non-treated and treated cells. Last of all, the effects detected in non-treated cells have to be considered for treated cells as well, so that the caffeine effect on its own can be accurately measured.

The results reported consist of an integrative approach chosen for the analysis of this data. By using this approach, we can evaluate the time-dependent effect of caffeine, comparing the expression profiles of cells exposed to lower doses (3 µM) and higher doses (10 µM) after 1, 3 and 9 hours with the control cells sampled in the beginning of the experiment. We can also evaluate the dose-dependent response and remove the background variations.

After 1 hour, 3 µM-treated cells appear to have increased activation of B and T cell receptors and toll-like receptors. Fos proto-oncogene (*Fos*), a gene that codes for a subunit of AP-1 transcription factor complex, is involved in all 3 processes mentioned above. Four tag clusters were associated with this gene and the expression of two of them is exclusively induced after cells are exposed to 3 µM of caffeine during 1 hour. One of these tag clusters is the one that maps closer to the known start of the gene. Other 3 tag clusters might indicate alternative transcription start sites for the expression of this gene. For the 3 pathways mentioned, upregulation of *Fos* indicates an increase in inflammatory and chemotactic events that, ultimately, lead to complement and coagulation activation. Upregulation of *Fos* and another member of the same family – *FosB* – indicate increase in osteoclast differentiation

processes. However, given the cell model and the ubiquitous nature of these genes, this might not be true.

Similarly to *Fos*, JunB proto-oncogene (*JunB*), also a subunit of AP-1 transcription factor complex, is perturbed by treatment with 10 µM of caffeine after 1 hour of exposure. This gene shows around 17 times more expression in cells that experience higher dose caffeine treatment and it is exclusively differentially expressed with caffeine treatment. These genes (*Fos* and *JunB*) are two of several immediate early response (IER) genes and have been shown to be induced by caffeine treatment before (Svenningsson *et al*., 1995). Concordantly with previous studies, our results show that physiological concentrations of caffeine can induce expression of IER genes. The list of IER genes was obtained from the literature search presented by a previous study (Arner *et al*., 2015). No enhancer was found to be regulating either of these genes.

Gap junction pathway is also found to be perturbed by 3 µM of caffeine exposure for 1 hour. *Tuba1* is the only perturbed gene associated with this pathway and is around 15 times less expression when caffeine is present. These results suggest that lower doses of caffeine inhibit extracellular matrix organization and cell interaction by gap junction assembly.

Synaptic activity seems to be very prominent after 1 hour of caffeine exposure in a lower dosage due to the upregulation of immediate early genes (*Egr1*, *Egr2* and *Egr3*). Neurotransmission processes seem to lack basic supply of acetyl co-A and, ultimately, can result in the decrease of neurotransmitters availability. Dopaminergic neurotransmission might be increased due to downregulation of *Nat8l* that leads to decrease of dopamine uptake and, thus, this neurotransmitter accumulates in synapses prolonging its excitatory activity (Arun *et al*., 2009; Moffet *et al*., 2013).

Overall, expression changes seem to be less and less evident over time in 3 µM-treated cells and non-existing for 10 µM-treated cells after 9 hours. We see that cells that underwent caffeine treatment show similar expression profiles after 9 hours as those of control cells. This might be explained by a lack of energy supply in the medium after a period of 9 hours in which cells have been moving resources to respond to caffeine stimulus. The medium was not changed during the period of the experiment, so no more glucose was added to it.

Expression of transcribed enhancers is usually very low, compared to expression of protein-coding genes, for instance (Andersson *et al*., 2014; Arner *et al*., 2015). Apart from few highly expressed enhancers, we also detected low eRNA expression levels. Assessing differences in expression of such lowly expressed genomic regions represents a challenge, mainly because no specific model has been developed for this end. Possibly because of this, the number of enhancers found to be differentially expressed and, thus, involved in caffeine response is very small. However, with good shape and correlation with putative target promoters, the enhancers we report give some important insights on regulation of gene-mediated response to caffeine.

For some enhancer-promoter pairs, the tag cluster representing that promoter is differentially expressed, but the enhancer is not. This might be explained by the fact that low levels of expression shown by most enhancers hampers the identification of slight changes in gene expression. Enhancers activity is time and cell-type specific (Maston *et al*., 2006). Given the heterogeneity of cell-types present in the cell population and presumable differences in differentiation stages of the same cells enhancer expression might be very different within the cell population.

Higher doses of caffeine appear to perturb immune response, extracellular matrix organization, endocytosis, axon guidance and neuroactive interaction between ligand and receptor. 10 µM of caffeine downregulates the expression of genes involved in generation of new neurons, neurogenesis and neuron differentiation.

Complement component 5a receptor 1 (*C5ar1*) is involved in the activation of the complement system by recognition of the complement component 5a. This receptor is expressed in neuronal cells and is involved in neuroinflammation processes. A 2.64 decrease in expression was detected in 10 µM-treated cells after 3 hours, when compared to non-treated cells at the point of time. Caffeine appears to inhibit the expression of this gene by shutting down the expression of one possible regulator enhancer. The expression of both enhancer and *C5ar1* promoter follow a similar pattern of gradually lower expression with the increase of caffeine concentration. The absence of eRNA seems to have significant effects on the promoter expression suggesting that this interaction is likely to occur *in vivo*. Coagulation cascade events appear to be decreased due to downregulation of two genes: plasminogen activator (*Plat*) and alpha-2 macroglobulin (*A2m*). However, no enhancer was found to be potential regulator of the promoters of both genes. Our results suggest that caffeine might have a neuroprotective role in the nervous system, by minimizing the activity of complement cascade events and possibly by minimizing the release of coagulation factors.

Sex determining region Y-box 2 (*Sox2*) shows very prominent expression after 3 hours in all 3 conditions, with higher levels in cells treated with 10 µM of caffeine. This gene is known to play a key role in somatic cell reprogramming by participating in reversing the differentiation stage of somatic cells to an embryonic-like pluripotency stage (Zhao *et al*., 2005; Zhang and Cui, 2014). It is also involved in differentiation of neuronal progenitors from pluripotent stem cells and maintenance of neuronal progenitors' stem cell potential. In the present study lt-NES cells, derived from iPSCs, were differentiated into a neuronal cell lineage, consisting of a mixture of neurons and glial cells. Although most cells showed a differentiated phenotype, a fraction of the cell population is expected to include undifferentiated cells and still differentiating cells. In both cases, Sox2 might be actively expressed. The changes observed for this gene might be representative of the cell population fraction that is not fully differentiated. The expression of the enhancer that was found to be associated with *Sox2* promoter shows good correlation with the expression of the promoter which indicates that the promoter is regulated by this enhancer. Detection of expression of genes involved in differentiation

processes suggests that the cell population is heterogeneous regarding differentiation stages. For specific, or follow-up, studies on neuronal gene expression, the differentiation stage of the cell population should be considered and a selection step could be included.

Sideways with its strengths, the study also shows limitations, which are key improvement points for follow-up or analogous studies. In order to improve the statistical significance of the results, the number of replicates should be increased. Performing the caffeine treatment more than once, in different weeks for instance, is also a proposal for inclusion of more biological replicates that can increase the statistical significance of the results. Since CAGE detects transcription initiation start sites by capturing capped mRNAs, this detection might be influenced by post-transcription re-capping events, which can limit the sensitivity of the technique (Li *et al*., 2016). mRNA re-capping events in the cytoplasm represent a mechanism of inactivation and reactivation of mRNA function and availability (Ignatochkina *et al*., 2015; Mukherjee *et al*., 2012; Kiss *et al*., 2015).

Overall, the results support our hypothesis that the response to caffeine is mediated by gene expression changes. These might occur downstream of the adenosine receptors mechanism inhibition. Our results indicate that caffeine regulates enhancer transcription as well. However, it is not clear if eRNA expression is a result of interaction with target promoter and, consequent, activation or eRNA expression triggers target promoter activation (Kim *et al*., 2015). Thus, we cannot conclude if the changes in enhancer expression we report are mediated by caffeine or if caffeine perturbs promoter expression by mechanisms other than eRNA regulation and enhancer expression is just a side response.

Our findings suggest that caffeine has subtle effects on gene expression, affecting only a small number of genes and these effects are not harmful or related to inflammatory responses of cell death. Lower and higher concentrations of caffeine appear to perturb different pathways and processes. The mechanism behind caffeine perturbation of gene expression seems to encompass, in part, perturbation of the regulatory mechanism exerted by enhancers.

7. Future Perspectives

Validation would be an essential complement to this study and, therefore, the next step. Analysis indicated putative enhancer-promoter pairs, which can be validated with high resolution chromatin conformation capture (Hi-C) as true interactions. Briefly, this method allows a genome-wide screen of proximity interactions between DNA regions that can be nearby each other or as far away as some enhancers are from the target promoter. The genomic regions in close proximity are captured together and then sequenced. A map of all genomic interactions is obtained and putative pairs can be validated as true interactions or not. With capture Hi-C, enrichment can be attained for the promoters we are interested in, greatly decreasing the number of interactions reported in the interaction map (Belton *et al*., 2012; Van Berkum *et al*., 2010).

Although extensive comparison between CAGE and qPCR has been conducted, qPCR could still be used to confirm the results and compare in terms of fold change.

Functional validation of enhancer-promoter pairs would greatly improve the study. Gain or loss of function experiments regarding the enhancers found to be involved in caffeine response and regulation of mediator genes can give valuable insights on the regulatory mechanisms of these mediator genes. If proven that eRNA has, in fact, a determining role for enhancer-mediated activation of target promoters, then interference RNA (iRNA) strategies could have the potential for future disruption of targeted gene expression and assessment of enhancer and promoter interaction mechanisms (Ren, 2010; Lai *et al*., 2013).

From the results obtained with this study, caffeine short-term effect on gene expression regarding physiological appears to be very subtle. Increasing the number of replicates could greatly improve the power of a study like this as well as facilitate the analysis of transcribed enhancers.

8. Conclusion

Caffeine is present in the everyday life of our society and economy. It attracts people with its ability to increase alertness, physical and cognitive performance and capability to develop work or other desired activity. Pharmacological effects in nervous and other body systems are known and, some of them, well characterized. This study showed that caffeine affects gene expression and stimulates or constrains several neuronal and immune processes in neuronal cells depending on dose and exposure time.

The study focuses on the effects of caffeine concerning the average daily consumption of caffeine-containing drinks such as coffee, tea and energetic drinks. With this in mind the concentrations of caffeine used in this study were physiological concentrations that correspond to 2-3 cups of coffee.

Our results provide strong foundation for other studies on how the human brain responds to caffeine and what enhancers regulate this response. This matter should still be left open for further studies and improved research. It is important to highlight the fact that no negative or dangerous effects were detected in this study. Caffeine does not appear to induce apoptosis or cell death pathways within the range of doses studied. Effect on gene expression appears to be very subtle, with no enormous changes detected. Numerous studies related to the nervous system, neurons and glial cells, nervous system development, neurodegenerative diseases or neurotoxicity studies can and should take advantage of differentiation of human neuronal cells from iPSCs.

These insights provide concrete hypotheses of physiological processes and associated genes for guiding further functional validation experiments with the potential to give valuable insights into the effects of caffeine, in a broader perspective, to the human brain.

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10. Supplementary Material

Supplementary Table 1 – Differentially expression results for all comparisons analyzed. Differentially expressed tag clusters are displayed with information on the correspondent comparison and the associated gene. Also shown are the logarithm of fold change (log_2FC) and statistical significance parameters.

TC	Gene Symbol	log_2FC	p-value	FDR	Regulation	Contrast
chr12:52445226..52445232,+	NR4A1	5,2032	3,7527E-15	1,3205E-10	up	0μM-1h|0μM-0h
chr5:137801166..137801178,+	EGR1	3,9756	1,7282E-14	3,0407E-10	up	0μM-1h|0μM-0h
chr6:166401410..166401570,-	LINC00473	3,1693	2,6212E-14	3,0746E-10	up	0μM-1h|0μM-0h
chr14:75746746..75746799,+	FOS	3,4549	2,3656E-09	2,0810E-05	up	0μM-1h|0μM-0h
chr5:137801721..137801731,+	EGR1	6,3454	3,3923E-09	2,3874E-05	up	0μM-1h|0μM-0h
chr19:45971254..45971258,+	FOSB	3,4856	2,1963E-06	1,2881E-02	up	0μM-1h|0μM-0h
chr14:75746345..75746363,+	FOS	1,6511	4,0479E-06	2,0349E-02	up	0μM-1h|0μM-0h
chr1:117142604..117142638,-	IGSF3	6,2814	6,5426E-06	2,8778E-02	up	0μM-1h|0μM-0h
chr12:89746177..89746295,-	DUSP6	1,2360	1,0224E-05	3,9975E-02	up	0μM-1h|0μM-0h
chr17:38506986..38507003,+	RARA	5,2045	1,2042E-05	4,2373E-02	up	0μM-1h|0μM-0h
chr5:74632605..74632742,-	CTD-2235C13.2	2,0963	1,5324E-05	4,6800E-02	up	0μM-1h|0μM-0h
chr13:102069583..102069593,-	None	3,6039	1,5959E-05	4,6800E-02	up	0μM-1h|0μM-0h
chr8:143696989..143697005,+	None	3,6329	1,7701E-05	4,7915E-02	up	0μM-1h|0μM-0h
chr11:66188506..66188510,+	NPAS4	4,1633	2,2816E-05	5,3798E-02	up	0μM-1h|0μM-0h
chr19:13263829..13263909,+	IER2	1,2640	2,4248E-05	5,3798E-02	up	0μM-1h|0μM-0h
chr3:79816991..79816992,-	ROBO1	4,8699	2,4461E-05	5,3798E-02	up	0μM-1h|0μM-0h
chr3:47932564..47932566,-	MAP4	3,5641	3,0505E-05	6,3144E-02	up	0μM-1h|0μM-0h
chr17:38375421..38375448,-	None	2,1875	4,4974E-05	8,5469E-02	up	0μM-1h|0μM-0h
chr5:137802527..137802551,-	None	3,2115	4,6148E-05	8,5469E-02	up	0μM-1h|0μM-0h
chr3:147127922..147127932,+	ZIC1	3,0305	7,1141E-11	2,5034E-06	up	0μM-3h|0μM-0h
chr6:166401410..166401570,-	LINC00473	2,5075	8,3687E-10	1,4724E-05	up	0μM-3h|0μM-0h
chr20:50179375..50179416,-	NFATC2	4,5872	1,9304E-09	2,2643E-05	up	0μM-3h|0μM-0h
chr19:45971254..45971258,+	FOSB	4,2882	4,2593E-09	3,7470E-05	up	0μM-3h|0μM-0h
chr19:54041677..54041684,+	ZNF331	5,0073	3,1816E-08	2,2391E-04	up	0μM-3h|0μM-0h
chr17:7747519..7747522,+	KDM6B	3,1991	9,7055E-08	5,6921E-04	up	0μM-3h|0μM-0h
chr13:102069583..102069593,-	None	4,1373	1,3148E-07	6,4214E-04	up	0μM-3h|0μM-0h
chr13:80912984..80913112,-	SPRY2	1,6158	1,4599E-07	6,4214E-04	up	0μM-3h|0μM-0h
chr1:90287518..90287585,+	LRRC8D	2,3574	2,0079E-07	6,8392E-04	up	0μM-3h|0μM-0h
chr12:52445226..52445232,+	NR4A1	3,5468	2,1136E-07	6,8392E-04	up	0μM-3h|0μM-0h
chr4:174255445..174255602,-	HMGB2	1,2816	2,1379E-07	6,8392E-04	up	0μM-3h|0μM-0h
chr2:237075077..237075158,-	GBX2	1,7504	2,9053E-07	8,5196E-04	up	0μM-3h|0μM-0h
chr22:38597973..38598117,+	MAFF	2,2599	3,8440E-07	1,0405E-03	up	0μM-3h|0μM-0h
chr17:46621281..46621290,-	HOXB2	1,8231	5,3513E-07	1,3451E-03	up	0μM-3h|0μM-0h
chr3:181429891..181429902,+	SOX2	2,1438	8,5860E-07	2,0142E-03	up	0μM-3h|0μM-0h
chr10:112257625..112257665,+	DUSP5	2,2727	1,0241E-06	2,2524E-03	up	0μM-3h|0μM-0h
chr16:3071261..3071269,+	TNFRSF12A	5,2306	1,9134E-06	3,9606E-03	up	0μM-3h|0μM-0h
chr5:137801166..137801178,+	EGR1	2,2415	3,2651E-06	6,3831E-03	up	0μM-3h|0μM-0h
chr6:26235190..26235245,-	HIST1H1D	1,1644	4,8852E-06	9,0477E-03	up	0μM-3h|0μM-0h
chr9:22009244..22009384,-	CDKN2B	1,7448	5,8654E-06	1,0320E-02	up	0μM-3h|0μM-0h
chr12:50158014..50158030,+	TMBIM6	4,4168	7,2493E-06	1,2147E-02	up	0μM-3h|0μM-0h
chr15:38545343..38545363,+	SPRED1	3,8375	9,9849E-06	1,5971E-02	up	0μM-3h|0μM-0h
chr17:61823031..61823032,-	RP11-51F16.8	-6,1411	1,2221E-05	1,8698E-02	down	0μM-3h|0μM-0h
chr20:36147678..36147711,-	BLCAP	-2,4771	1,3701E-05	2,0089E-02	down	0μM-3h|0μM-0h
chr4:73259528..73259558,-	ADAMTS3	7,2818	1,4930E-05	2,0928E-02	up	0μM-3h|0μM-0h
chr14:75746345..75746363,+	FOS	1,5162	1,5463E-05	2,0928E-02	up	0μM-3h|0μM-0h
chr14:75746746..75746799,+	FOS	2,4426	1,8817E-05	2,4524E-02	up	0μM-3h|0μM-0h
chr19:47793142..47793248,+	C5AR1	1,5137	2,8083E-05	3,5293E-02	up	0μM-3h|0μM-0h
chr4:4393197..4393249,+	NSG1	-2,3406	3,4461E-05	3,9939E-02	down	0μM-3h|0μM-0h
chr15:92981585..92981785,+	ST8SIA2	-2,7957	3,5801E-05	3,9939E-02	down	0μM-3h|0μM-0h
chr9:107690783..107690836,-	ABCA1	-2,6062	3,6875E-05	3,9939E-02	down	0μM-3h|0μM-0h
chr12:49582229..49582231,-	TUBA1A	2,9532	3,6916E-05	3,9939E-02	up	0μM-3h|0μM-0h
chr10:64576110..64576126,-	EGR2	4,2955	3,7454E-05	3,9939E-02	up	0μM-3h|0μM-0h

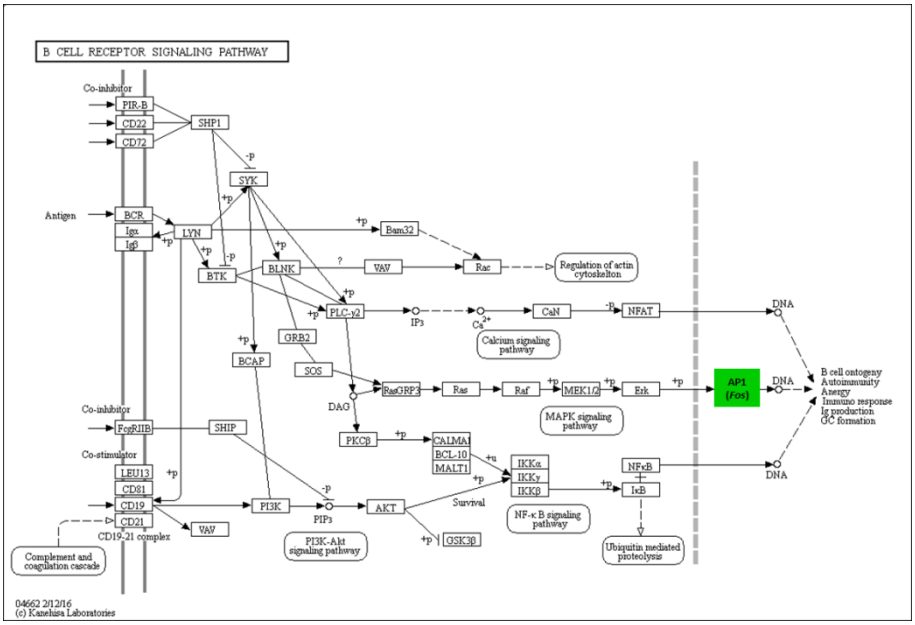
chr19:1203161..1203164,+	STK11	5,7946	4,6232E-05	4,7849E-02	up	0µM-3h|0µM-0h
chr12:17143909..17143931,+	None	-2,7680	5,1267E-05	5,1544E-02	down	0µM-3h|0µM-0h
chr4:74904404..74904523,-	CXCL3	1,6633	5,4626E-05	5,3395E-02	up	0µM-3h|0µM-0h
chr7:1542698..1542824,-	INTS1	-2,7186	5,9057E-05	5,6166E-02	down	0µM-3h|0µM-0h
chr11:134095347..134095354,-	NCAPD3	3,6841	6,3383E-05	5,8694E-02	up	0µM-3h|0µM-0h
chr17:7741660..7741714,+	None	-1,1428	8,5524E-05	7,7155E-02	down	0µM-3h|0µM-0h
chr13:78477332..78477399,-	EDNRB	-2,5019	8,7704E-05	7,7155E-02	down	0µM-3h|0µM-0h
chr22:39639967..39639983,-	PDGFB	2,0469	1,0713E-04	9,1947E-02	up	0µM-3h|0µM-0h
chr5:137801721..137801731,+	EGR1	4,2762	1,0991E-04	9,2087E-02	up	0µM-3h|0µM-0h
chr2:122097376..122097402,-	CLASP1	-5,5178	1,1511E-04	9,2266E-02	down	0µM-3h|0µM-0h
chr5:55800743..55800940,-	None	-4,0093	1,1537E-04	9,2266E-02	down	0µM-3h|0µM-0h
chr19:13263829..13263909,+	IER2	1,1481	1,1870E-04	9,2821E-02	up	0µM-3h|0µM-0h
chr1:156084459..156084586,+	LMNA	1,3773	1,3304E-04	9,9669E-02	up	0µM-3h|0µM-0h
chr17:17399706..17399709,-	RASD1	1,7490	1,3545E-04	9,9669E-02	up	0µM-3h|0µM-0h
chr6:26056567..26056704,-	HIST1H1C	1,3806	1,3596E-04	9,9669E-02	up	0µM-3h|0µM-0h
chr7:1272476..1272536,+	UNCX	-1,5781	5,0296E-08	1,7699E-03	down	0µM-9h|0µM-0h
chr6:33378449..33378483,+	PHF1	-1,6457	4,8325E-07	8,5025E-03	down	0µM-9h|0µM-0h
chr1:27693431..27693471,-	MAP3K6	-2,4568	1,4874E-06	1,7447E-02	down	0µM-9h|0µM-0h
chr6:36164969..36164979,-	RP1-179N16.6	-3,8840	3,2102E-06	2,1268E-02	down	0µM-9h|0µM-0h
chr17:42200961..42201050,-	HDAC5	-1,1581	3,5261E-06	2,1268E-02	down	0µM-9h|0µM-0h
chr18:8705551..8705612,+	SOGA2	-1,5521	3,6264E-06	2,1268E-02	down	0µM-9h|0µM-0h
chr6:166401410..166401570,-	LINC00473	1,8720	5,4344E-06	2,4163E-02	up	0µM-9h|0µM-0h
chr9:96214192..96214228,+	FAM120A	1,3444	5,4933E-06	2,4163E-02	up	0µM-9h|0µM-0h
chr8:80577655..80577658,+	STMN2	3,3015	6,4248E-06	2,5120E-02	up	0µM-9h|0µM-0h
chr12:50158014..50158030,+	TMBIM6	4,4283	8,3764E-06	2,9476E-02	up	0µM-9h|0µM-0h
chr2:220435334..220435365,+	INHA	-2,4505	9,5424E-06	3,0526E-02	down	0µM-9h|0µM-0h
chrX:54522665..54522666,-	FGD1	-1,9013	1,1022E-05	3,1925E-02	down	0µM-9h|0µM-0h
chr19:18530187..18530270,+	SSBP4	-1,2132	1,1830E-05	3,1925E-02	down	0µM-9h|0µM-0h
chr1:117142604..117142638,-	IGSF3	6,0262	1,2701E-05	3,1925E-02	up	0µM-9h|0µM-0h
chr13:102069583..102069593,-	None	3,5680	1,4354E-05	3,3674E-02	up	0µM-9h|0µM-0h
chr17:7307971..7307980,+	NLGN2	-1,7777	1,7180E-05	3,7785E-02	down	0µM-9h|0µM-0h
chr20:57466441..57466479,+	GNAS	-1,0984	1,9883E-05	4,1156E-02	down	0µM-9h|0µM-0h
chr9:128508946..128509011,+	None	-1,1303	2,5542E-05	4,8936E-02	down	0µM-9h|0µM-0h
chr1:155100330..155100396,+	EFNA1	-1,5604	2,6422E-05	4,8936E-02	down	0µM-9h|0µM-0h
chr20:60640871..60640939,-	TAF4	-1,3045	2,8522E-05	5,0183E-02	down	0µM-9h|0µM-0h
chr16:28875116..28875190,+	SH2B1	-2,0415	3,2278E-05	5,2645E-02	down	0µM-9h|0µM-0h
chr16:56659693..56659694,+	MT1E	2,5236	3,2914E-05	5,2645E-02	up	0µM-9h|0µM-0h
chr2:172016919..172016950,-	TLK1	1,8689	4,0912E-05	6,0411E-02	up	0µM-9h|0µM-0h
chr10:92680843..92680847,-	ANKRD1	-3,6079	4,1553E-05	6,0411E-02	down	0µM-9h|0µM-0h
chr21:45553353..45553370,-	None	-6,3278	4,2919E-05	6,0411E-02	down	0µM-9h|0µM-0h
chr16:29817671..29817684,+	MAZ	-3,2472	4,6309E-05	6,2675E-02	down	0µM-9h|0µM-0h
chr17:34058645..34058704,+	RASL10B	-1,3166	5,1463E-05	6,7072E-02	down	0µM-9h|0µM-0h
chr9:134346213..134346245,-	None	-2,7114	5,7719E-05	7,1072E-02	down	0µM-9h|0µM-0h
chr20:3827370..3827388,-	None	-3,3089	5,8572E-05	7,1072E-02	down	0µM-9h|0µM-0h
chr11:32914355..32914371,+	QSER1	-1,5636	6,6184E-05	7,3784E-02	down	0µM-9h|0µM-0h
chr12:133066715..133066774,+	FBRSL1	-1,7078	6,6539E-05	7,3784E-02	down	0µM-9h|0µM-0h
chr17:79995113..79995116,-	DCXR	3,3147	6,7097E-05	7,3784E-02	up	0µM-9h|0µM-0h
chr14:100437728..100437913,+	EVL	-1,2841	7,2735E-05	7,7560E-02	down	0µM-9h|0µM-0h
chr4:95129064..95129065,+	SMARCAD1	6,0481	7,6289E-05	7,8956E-02	up	0µM-9h|0µM-0h
chr1:1475973..1476003,-	TMEM240	-1,6149	8,1023E-05	8,0590E-02	down	0µM-9h|0µM-0h
chr8:72448325..72448332,-	RP11-1102P16.1	3,3703	8,2448E-05	8,0590E-02	up	0µM-9h|0µM-0h
chr12:6930948..6931043,+	GPR162	-1,1947	8,5144E-05	8,0764E-02	down	0µM-9h|0µM-0h
chr19:46220508..46220585,-	FBXO46	-2,0491	8,7216E-05	8,0764E-02	down	0µM-9h|0µM-0h
chr7:143077435..143077523,+	None	-1,3229	9,3391E-05	8,4265E-02	down	0µM-9h|0µM-0h
chr3:147127922..147127932,+	ZIC1	1,8003	1,0018E-04	8,8131E-02	up	0µM-9h|0µM-0h
chr7:100810853..100810866,-	None	1,5560	1,0598E-04	9,0266E-02	up	0µM-9h|0µM-0h
chr6:44238177..44238191,+	TMEM151B	-1,3737	1,0800E-04	9,0266E-02	down	0µM-9h|0µM-0h
chr1:185285480..185285489,+	None	5,8428	1,1030E-04	9,0266E-02	up	0µM-9h|0µM-0h
chr20:62711332..62711464,+	OPRL1	-1,6292	1,1815E-04	9,4490E-02	down	0µM-9h|0µM-0h
chr2:219524198..219524201,-	ZNF142	4,7757	1,2547E-04	9,6571E-02	up	0µM-9h|0µM-0h
chr10:102790986..102791052,+	SFXN3	-1,0863	1,2624E-04	9,6571E-02	down	0µM-9h|0µM-0h

chr5:137801166..137801178,+	EGR1	4,6356	1,0344E-18	3,6399E-14	up	10µM-1h|0µM-0h
chr12:52445226..52445232,+	NR4A1	5,5359	4,8420E-18	8,5192E-14	up	10µM-1h|0µM-0h
chr14:75746746..75746799,+	FOS	4,7494	2,5954E-16	3,0444E-12	up	10µM-1h|0µM-0h
chr19:45971254..45971258,+	FOSB	4,8753	1,3909E-11	1,2236E-07	up	10µM-1h|0µM-0h
chr5:137801721..137801731,+	EGR1	7,0491	2,2679E-11	1,5207E-07	up	10µM-1h|0µM-0h
chr6:166401410..166401570,-	LINC00473	2,6486	2,5929E-11	1,5207E-07	up	10µM-1h|0µM-0h
chr19:13263829..13263909,+	IER2	1,8451	2,6222E-10	1,3182E-06	up	10µM-1h|0µM-0h
chr5:137802527..137802551,-	None	4,7445	2,0342E-09	8,9477E-06	up	10µM-1h|0µM-0h
chr11:66188506..66188510,+	NPAS4	5,6691	4,9599E-09	1,9393E-05	up	10µM-1h|0µM-0h
chr10:64576110..64576126,-	EGR2	6,0177	1,7472E-08	6,1483E-05	up	10µM-1h|0µM-0h
chr19:39897487..39897490,+	ZFP36	2,3933	7,8919E-08	2,5246E-04	up	10µM-1h|0µM-0h
chr19:2476120..2476127,+	GADD45B	1,6857	8,7102E-07	2,5542E-03	up	10µM-1h|0µM-0h
chr14:75746345..75746363,+	FOS	1,6071	1,9568E-06	5,2966E-03	up	10µM-1h|0µM-0h
chr19:54041677..54041684,+	ZNF331	4,3218	3,6149E-06	9,0861E-03	up	10µM-1h|0µM-0h
chr8:22550990..22550992,-	EGR3	4,9801	5,5332E-06	1,2980E-02	up	10µM-1h|0µM-0h
chr12:89746177..89746295,-	DUSP6	1,2006	6,3821E-06	1,4036E-02	up	10µM-1h|0µM-0h
chr13:102069583..102069593,-	None	3,5174	8,4119E-06	1,6497E-02	up	10µM-1h|0µM-0h
chr10:112257625..112257665,+	DUSP5	1,9979	8,4387E-06	1,6497E-02	up	10µM-1h|0µM-0h
chr14:75747308..75747348,+	FOS	3,9872	9,4812E-06	1,7560E-02	up	10µM-1h|0µM-0h
chr12:50158014..50158030,+	TMBIM6	4,2637	1,0827E-05	1,9050E-02	up	10µM-1h|0µM-0h
chr18:72163641..72163644,+	CNDP2	3,1666	1,1623E-05	1,9476E-02	up	10µM-1h|0µM-0h
chr19:12902296..12902300,+	JUNB	4,1025	1,6676E-05	2,6467E-02	up	10µM-1h|0µM-0h
chr8:143696989..143697005,+	None	3,4854	1,7299E-05	2,6467E-02	up	10µM-1h|0µM-0h
chr5:137801304..137801312,+	EGR1	4,6467	3,0286E-05	4,4405E-02	up	10µM-1h|0µM-0h
chr6:166401410..166401570,-	LINC00473	3,4782	8,8514E-17	3,1147E-12	up	10µM-3h|0µM-0h
chr19:54041677..54041684,+	ZNF331	6,6420	8,6392E-15	1,5200E-10	up	10µM-3h|0µM-0h
chr19:45971254..45971258,+	FOSB	4,0624	4,2281E-08	4,9595E-04	up	10µM-3h|0µM-0h
chr12:52445226..52445232,+	NR4A1	3,6001	4,5897E-07	4,0376E-03	up	10µM-3h|0µM-0h
chr7:100808717..100808856,-	VGF	1,3074	1,1898E-06	7,8221E-03	up	10µM-3h|0µM-0h
chr1:117142604..117142638,-	IGSF3	6,6032	1,5516E-06	7,8221E-03	up	10µM-3h|0µM-0h
chr11:66176517..66176658,+	RP11-867G23.10	1,4248	1,6910E-06	7,8221E-03	up	10µM-3h|0µM-0h
chr3:181430136..181430158,+	SOX2	2,2770	1,7783E-06	7,8221E-03	up	10µM-3h|0µM-0h
chr13:102069583..102069593,-	None	3,8778	2,1963E-06	8,5873E-03	up	10µM-3h|0µM-0h
chr11:47587007..47587033,+	PTPMT1	-3,1572	3,5087E-06	1,1438E-02	down	10µM-3h|0µM-0h
chr2:74347689..74347702,-	None	2,2914	3,5756E-06	1,1438E-02	up	10µM-3h|0µM-0h
chr17:76356151..76356245,-	SOCS3	-2,4830	4,3333E-06	1,2707E-02	down	10µM-3h|0µM-0h
chr10:112257625..112257665,+	DUSP5	2,1410	5,4779E-06	1,4828E-02	up	10µM-3h|0µM-0h
chr1:155100330..155100396,+	EFNA1	-1,7034	6,9913E-06	1,7573E-02	down	10µM-3h|0µM-0h
chr1:90287518..90287585,+	LRRC8D	2,0456	8,0495E-06	1,8884E-02	up	10µM-3h|0µM-0h
chr1:68849933..68849989,-	None	-3,0006	9,4051E-06	2,0685E-02	down	10µM-3h|0µM-0h
chr9:116263519..116263529,+	RGS3	-2,6682	1,0849E-05	2,2457E-02	down	10µM-3h|0µM-0h
chr1:155036179..155036245,+	EFNA4	-1,4048	1,3215E-05	2,5835E-02	down	10µM-3h|0µM-0h
chr2:73511412..73511416,-	FBXO41	3,5747	1,4219E-05	2,6335E-02	up	10µM-3h|0µM-0h
chr1:230849855..230849885,-	RP11-99J16__A.2	-3,0005	1,8502E-05	3,2553E-02	down	10µM-3h|0µM-0h
chr9:117880507..117880508,-	TNC	-4,2949	2,0356E-05	3,3451E-02	down	10µM-3h|0µM-0h
chr10:32635948..32636143,-	AL391839.1	1,0146	2,0913E-05	3,3451E-02	up	10µM-3h|0µM-0h
chr8:41045184..41045185,+	None	-3,9508	3,2741E-05	5,0093E-02	down	10µM-3h|0µM-0h
chr14:75746345..75746363,+	FOS	1,4873	3,4921E-05	5,1201E-02	up	10µM-3h|0µM-0h
chr1:170632299..170632303,+	PRRX1	-6,3733	3,7061E-05	5,2166E-02	down	10µM-3h|0µM-0h
chr17:33935215..33935361,+	AP2B1	-3,2476	3,8971E-05	5,2745E-02	down	10µM-3h|0µM-0h
chr5:176738893..176738928,-	MXD3	-1,2640	4,7415E-05	5,9414E-02	down	10µM-3h|0µM-0h
chr4:95129064..95129065,+	SMARCAD1	6,2807	4,8061E-05	5,9414E-02	up	10µM-3h|0µM-0h
chr18:3594147..3594151,+	DLGAP1-AS1	-1,8677	4,8965E-05	5,9414E-02	down	10µM-3h|0µM-0h
chr17:38506986..38507003,+	RARA	4,7649	6,1479E-05	7,0877E-02	up	10µM-3h|0µM-0h
chr19:1203161..1203164,+	STK11	5,7785	6,2440E-05	7,0877E-02	up	10µM-3h|0µM-0h
chr4:174255445..174255602,-	HMGB2	0,9862	6,8172E-05	7,3467E-02	up	10µM-3h|0µM-0h
chr4:1988054..1988078,-	MIR943	-6,0178	6,8936E-05	7,3467E-02	down	10µM-3h|0µM-0h
chr7:131708315..131708348,-	None	-6,3732	7,0984E-05	7,3467E-02	down	10µM-3h|0µM-0h
chr15:38545343..38545363,+	SPRED1	3,4861	7,3771E-05	7,4170E-02	up	10µM-3h|0µM-0h
chr8:8185427..8185428,-	SGK223	-6,2495	7,9647E-05	7,7853E-02	down	10µM-3h|0µM-0h
chr12:50158014..50158030,+	TMBIM6	4,0916	8,3832E-05	7,9728E-02	up	10µM-3h|0µM-0h

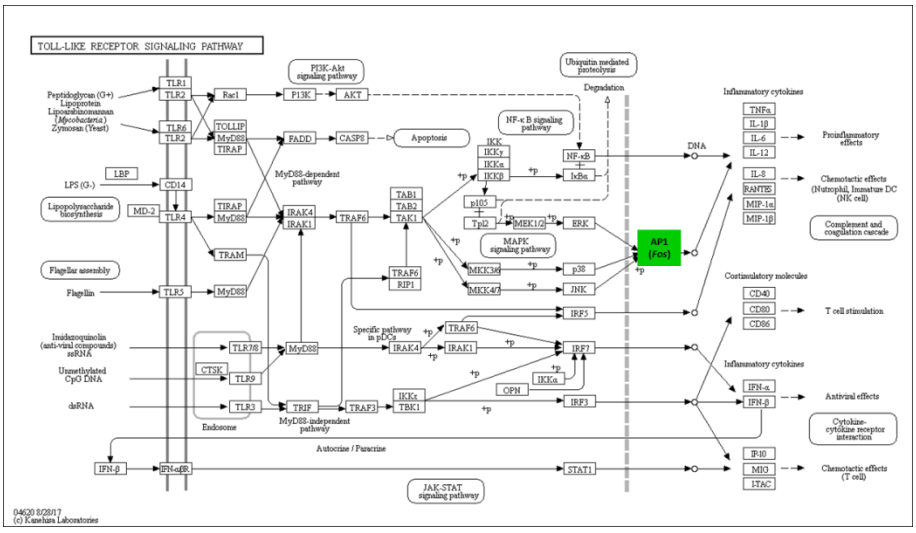
chr3:147127922..147127932,+	ZIC1	1,8595	8,8928E-05	8,1210E-02	up	10µM-3h|0µM-0h
chr2:114082650..114082829,+	None	1,5445	9,0005E-05	8,1210E-02	up	10µM-3h|0µM-0h
chr14:75746746..75746799,+	FOS	2,2907	9,6322E-05	8,4736E-02	up	10µM-3h|0µM-0h
chr14:23451703..23451850,-	AJUBA	-1,4279	1,0134E-04	8,6979E-02	down	10µM-3h|0µM-0h
chr16:30960375..30960444,+	FBXL19	-1,3074	1,0975E-04	9,1953E-02	down	10µM-3h|0µM-0h
chr12:112856638..112856655,-	RPL6	4,5638	1,1295E-04	9,2431E-02	up	10µM-3h|0µM-0h
chr1:230849855..230849885,-	RP11-99J16__A.2	-4,0665	1,7567E-08	6,1816E-04	down	10µM-3h|0µM-3h
chr11:33912734..33912904,-	LMO2	-1,9604	4,0917E-07	4,8581E-03	down	10µM-3h|0µM-3h
chr1:68849933..68849989,-	None	-3,4993	4,1417E-07	4,8581E-03	down	10µM-3h|0µM-3h
chr4:1166553..1166632,-	SPON2	-2,0633	2,2472E-06	1,4702E-02	down	10µM-3h|0µM-3h
chr8:41045184..41045185,+	None	-4,4372	2,2773E-06	1,4702E-02	down	10µM-3h|0µM-3h
chr11:47587007..47587033,+	PTPMT1	-3,1902	2,5068E-06	1,4702E-02	down	10µM-3h|0µM-3h
chr9:117880507..117880508,-	TNC	-4,5244	5,5571E-06	2,4156E-02	down	10µM-3h|0µM-3h
chr19:48828781..48828845,+	EMP3	-1,4053	5,8579E-06	2,4156E-02	down	10µM-3h|0µM-3h
chr14:72399939..72399950,+	RGS6	-2,1218	6,1781E-06	2,4156E-02	down	10µM-3h|0µM-3h
chr9:85896499..85896549,-	FRMD3	-2,1216	7,9794E-06	2,5695E-02	down	10µM-3h|0µM-3h
chr9:117880803..117880810,-	TNC	-2,1165	8,0323E-06	2,5695E-02	down	10µM-3h|0µM-3h
chr14:27065822..27065829,-	NOVA1	1,9769	8,7925E-06	2,5783E-02	up	10µM-3h|0µM-3h
chr2:233498267..233498325,+	EFHD1	-1,8994	1,0017E-05	2,6708E-02	down	10µM-3h|0µM-3h
chr1:170632299..170632303,+	PRRX1	-6,6844	1,0626E-05	2,6708E-02	down	10µM-3h|0µM-3h
chr10:54073963..54074044,+	DKK1	-1,4999	1,1847E-05	2,7793E-02	down	10µM-3h|0µM-3h
chr11:66103870..66103944,-	RIN1	-1,5396	1,2734E-05	2,8006E-02	down	10µM-3h|0µM-3h
chr9:117880930..117880933,-	TNC	-3,1072	1,5703E-05	3,0005E-02	down	10µM-3h|0µM-3h
chr18:23879217..23879218,-	U3	-3,1788	1,6079E-05	3,0005E-02	down	10µM-3h|0µM-3h
chr3:197639715..197639883,-	IQCG	-1,9374	1,6201E-05	3,0005E-02	down	10µM-3h|0µM-3h
chr8:42065046..42065226,-	PLAT	-1,7521	1,9164E-05	3,3718E-02	down	10µM-3h|0µM-3h
chr7:131708315..131708348,-	None	-6,7273	2,1588E-05	3,6173E-02	down	10µM-3h|0µM-3h
chrM:3892..3893,-	MT-TQ	-3,3282	2,6512E-05	4,0867E-02	down	10µM-3h|0µM-3h
chr1:109513893..109513902,-	WDR47	4,7273	2,7324E-05	4,0867E-02	up	10µM-3h|0µM-3h
chr14:95078714..95078731,+	RP11-986E7.7	-3,4289	2,8775E-05	4,0867E-02	down	10µM-3h|0µM-3h
chr5:87688005..87688118,+	TMEM161B-AS1	-1,7197	2,9034E-05	4,0867E-02	down	10µM-3h|0µM-3h
chr22:30792980..30793115,+	SEC14L2	-1,2519	3,2399E-05	4,2804E-02	down	10µM-3h|0µM-3h
chr11:64055836..64055880,+	GPR137	-2,4804	3,2843E-05	4,2804E-02	down	10µM-3h|0µM-3h
chr18:3594414..3594460,+	DLGAP1-AS1	-1,3482	3,4813E-05	4,3751E-02	down	10µM-3h|0µM-3h
chr5:82155700..82155782,-	None	-1,7704	3,6317E-05	4,4067E-02	down	10µM-3h|0µM-3h
chr8:108510073..108510145,-	ANGPT1	-2,5243	3,9228E-05	4,6013E-02	down	10µM-3h|0µM-3h
chr9:118024730..118024837,-	None	-2,3625	4,4357E-05	5,0251E-02	down	10µM-3h|0µM-3h
chr2:122097376..122097402,-	CLASP1	5,8531	4,5697E-05	5,0251E-02	up	10µM-3h|0µM-3h
chr16:66638575..66638661,+	CMTM3	-1,2380	5,0846E-05	5,4219E-02	down	10µM-3h|0µM-3h
chr14:70497271..70497289,+	SMOC1	-1,6759	5,8564E-05	6,0612E-02	down	10µM-3h|0µM-3h
chr1:161123863..161123864,+	UFC1	-6,2461	7,0230E-05	7,0156E-02	down	10µM-3h|0µM-3h
chr11:4414897..4414944,-	TRIM21	-1,4002	7,1773E-05	7,0156E-02	down	10µM-3h|0µM-3h
chr8:30242022..30242057,+	RBPMS	-3,4113	8,9590E-05	8,5205E-02	down	10µM-3h|0µM-3h
chr2:47235797..47235841,+	TTC7A	-1,9232	9,8079E-05	8,6494E-02	down	10µM-3h|0µM-3h
chr5:146939416..146939577,+	None	-2,4748	1,0051E-04	8,6494E-02	down	10µM-3h|0µM-3h
chr11:27062907..27062948,+	BBOX1	-2,9845	1,0186E-04	8,6494E-02	down	10µM-3h|0µM-3h
chr5:152869973..152869989,+	GRIA1	-3,6837	1,0387E-04	8,6494E-02	down	10µM-3h|0µM-3h
chr12:49658859..49658867,+	TUBA1C	-1,5452	1,0608E-04	8,6494E-02	down	10µM-3h|0µM-3h
chr7:116166382..116166446,+	CAV1	-2,2879	1,0894E-04	8,6494E-02	down	10µM-3h|0µM-3h
chr11:74109183..74109218,-	PGM2L1	3,7624	1,1144E-04	8,6494E-02	up	10µM-3h|0µM-3h
chr8:82283530..82283531,+	None	-6,1904	1,1931E-04	8,6494E-02	down	10µM-3h|0µM-3h
chr10:86184670..86184734,+	CCSER2	-1,4134	1,2041E-04	8,6494E-02	down	10µM-3h|0µM-3h
chr1:161337654..161337702,-	C1orf192	-1,3161	1,2447E-04	8,6494E-02	down	10µM-3h|0µM-3h
chr12:9229984..9230010,-	A2M	-6,2073	1,2885E-04	8,6494E-02	down	10µM-3h|0µM-3h
chr18:10125244..10125298,+	RP11-419J16.1	-1,8985	1,3330E-04	8,6494E-02	down	10µM-3h|0µM-3h
chr1:15036253..15036398,+	KAZN	4,8101	1,3356E-04	8,6494E-02	up	10µM-3h|0µM-3h
chr7:7310912..7310949,+	AC005532.5	-6,1615	1,3619E-04	8,6494E-02	down	10µM-3h|0µM-3h
chr2:170590170..170590222,+	KLHL23	1,0725	1,3623E-04	8,6494E-02	up	10µM-3h|0µM-3h
chr19:13106245..13106369,+	NFIX	-1,5500	1,3795E-04	8,6494E-02	down	10µM-3h|0µM-3h
chr15:64126106..64126197,-	HERC1	1,2051	1,3803E-04	8,6494E-02	up	10µM-3h|0µM-3h
chr16:450354..450445,-	None	-1,6613	1,4020E-04	8,6494E-02	down	10µM-3h|0µM-3h

chr17:10741371..10741417,-	PIRT	-1,9591	1,4108E-04	8,6494E-02	down	10µM-3h|0µM-3h
chr14:79745720..79745742,+	NRXN3	1,0336	1,4288E-04	8,6494E-02	up	10µM-3h|0µM-3h
chr7:136912578..136912580,-	PTN	-6,4394	1,4469E-04	8,6494E-02	down	10µM-3h|0µM-3h
chr2:105320104..105320236,+	AC068057.1	-1,2294	1,4502E-04	8,6494E-02	down	10µM-3h|0µM-3h
chr16:4750968..4750969,-	ANKS3	2,2835	1,5532E-04	8,8523E-02	up	10µM-3h|0µM-3h
chr4:80994234..80994278,-	ANTXR2	-1,9472	1,5945E-04	8,8523E-02	down	10µM-3h|0µM-3h
chr7:148725499..148725502,-	PDIA4	-2,4839	1,5988E-04	8,8523E-02	down	10µM-3h|0µM-3h
chr19:47793142..47793248,+	C5AR1	-1,4045	1,6009E-04	8,8523E-02	down	10µM-3h|0µM-3h
chr19:46010674..46010790,+	VASP	-1,0513	1,6318E-04	8,8523E-02	down	10µM-3h|0µM-3h
chr1:155100330..155100396,+	EFNA1	-1,4233	1,6352E-04	8,8523E-02	down	10µM-3h|0µM-3h
chr5:111964555..111964559,+	RP11-159K7.2	-4,0908	1,7394E-04	9,1002E-02	down	10µM-3h|0µM-3h
chr12:13349608..13349739,+	EMP1	-1,3435	1,7664E-04	9,1002E-02	down	10µM-3h|0µM-3h
chr15:63449622..63449707,-	RPS27L	-1,4708	1,8183E-04	9,1002E-02	down	10µM-3h|0µM-3h
chr19:18451419..18451456,+	PGPEP1	-1,3736	1,8506E-04	9,1002E-02	down	10µM-3h|0µM-3h
chr17:19619854..19619980,-	SLC47A2	-2,1986	1,8545E-04	9,1002E-02	down	10µM-3h|0µM-3h
chr4:79892775..79892785,+	LINC01088	-4,5435	1,8565E-04	9,1002E-02	down	10µM-3h|0µM-3h
chr10:25304893..25305088,-	ENKUR	-1,1457	1,8691E-04	9,1002E-02	down	10µM-3h|0µM-3h
chr13:24144696..24144703,+	TNFRSF19	-2,2391	1,8879E-04	9,1002E-02	down	10µM-3h|0µM-3h
chr15:52312119..52312185,+	MAPK6	-1,6125	1,9881E-04	9,4542E-02	down	10µM-3h|0µM-3h
chr7:100464965..100465052,+	TRIP6	-1,2471	2,1041E-04	9,6418E-02	down	10µM-3h|0µM-3h
chr20:46414830..46414841,-	SULF2	1,2280	2,1218E-04	9,6418E-02	up	10µM-3h|0µM-3h
chr1:150230218..150230301,+	CA14	-1,3272	2,1369E-04	9,6418E-02	down	10µM-3h|0µM-3h
chr6:34544362..34544543,-	None	-1,4623	2,1597E-04	9,6418E-02	down	10µM-3h|0µM-3h
chr7:75909940..75909961,+	SRRM3	-5,9999	2,1646E-04	9,6418E-02	down	10µM-3h|0µM-3h
chr5:146268009..146268057,-	PPP2R2B	-3,4618	2,2184E-04	9,7156E-02	down	10µM-3h|0µM-3h
chr12:41221614..41221806,+	CNTN1	1,3413	2,2364E-04	9,7156E-02	up	10µM-3h|0µM-3h
chr8:125384631..125384642,-	TMEM65	-1,3604	2,3021E-04	9,8792E-02	down	10µM-3h|0µM-3h
chr1:1293908..1293956,-	MXRA8	-1,3579	2,3561E-04	9,9620E-02	down	10µM-3h|0µM-3h
chr6:90122426..90122440,-	RRAGD	-3,9477	2,3780E-04	9,9620E-02	down	10µM-3h|0µM-3h
chr13:102069583..102069593,-	None	4,0685	3,6670E-07	1,2904E-02	up	10µM-9h|0µM-0h
chr5:137801166..137801178,+	EGR1	4,0958	1,0159E-15	3,5750E-11	up	3µM-1h|0µM-0h
chr14:75746746..75746799,+	FOS	4,4311	1,3494E-14	2,3741E-10	up	3µM-1h|0µM-0h
chr12:52445226..52445232,+	NR4A1	4,8888	5,0204E-14	5,8888E-10	up	3µM-1h|0µM-0h
chr6:166401410..166401570,-	LINC00473	2,9034	5,3483E-13	4,7050E-09	up	3µM-1h|0µM-0h
chr19:45971254..45971258,+	FOSB	5,0858	2,5220E-12	1,7749E-08	up	3µM-1h|0µM-0h
chr5:137801721..137801731,+	EGR1	6,6197	3,2280E-10	1,8932E-06	up	3µM-1h|0µM-0h
chr13:102069583..102069593,-	None	4,4683	5,2749E-09	2,6517E-05	up	3µM-1h|0µM-0h
chr10:64576110..64576126,-	EGR2	5,7988	4,7686E-08	2,0975E-04	up	3µM-1h|0µM-0h
chr5:137802527..137802551,-	None	4,0234	2,2033E-07	8,4842E-04	up	3µM-1h|0µM-0h
chr14:75746345..75746363,+	FOS	1,7686	2,4110E-07	8,4842E-04	up	3µM-1h|0µM-0h
chr19:13263829..13263909,+	IER2	1,4664	3,4622E-07	1,1076E-03	up	3µM-1h|0µM-0h
chr11:66188506..66188510,+	NPAS4	4,7509	7,0775E-07	2,0754E-03	up	3µM-1h|0µM-0h
chr12:89746177..89746295,-	DUSP6	1,2617	2,3240E-06	6,2906E-03	up	3µM-1h|0µM-0h
chr12:50158014..50158030,+	TMBIM6	4,5982	2,6975E-06	6,7802E-03	up	3µM-1h|0µM-0h
chr1:96912447..96912464,+	None	-2,7486	9,5239E-06	2,2342E-02	down	3µM-1h|0µM-0h
chr4:2060965..2061039,+	NAT8L	-1,8570	1,8601E-05	3,9375E-02	down	3µM-1h|0µM-0h
chr14:75747308..75747348,+	FOS	3,8262	1,9200E-05	3,9375E-02	up	3µM-1h|0µM-0h
chr8:22550990..22550992,-	EGR3	4,7094	2,0141E-05	3,9375E-02	up	3µM-1h|0µM-0h
chr14:75745511..75745535,+	FOS	3,7600	2,2954E-05	4,2511E-02	up	3µM-1h|0µM-0h
chr12:49579459..49579461,-	TUBA1A	-3,9475	2,9478E-05	5,1859E-02	down	3µM-1h|0µM-0h
chr2:74687384..74687397,+	WBP1	-4,1459	3,2116E-05	5,1859E-02	down	3µM-1h|0µM-0h
chr11:32914355..32914371,+	QSER1	-1,5085	3,3306E-05	5,1859E-02	down	3µM-1h|0µM-0h
chr17:38506986..38507003,+	RARA	4,8353	3,3896E-05	5,1859E-02	up	3µM-1h|0µM-0h
chr1:85824476..85824535,-	DDAH1	-2,4028	4,2077E-05	6,1694E-02	down	3µM-1h|0µM-0h
chr7:142375408..142375410,+	MTRNR2L6	-3,0240	5,4115E-05	7,6170E-02	down	3µM-1h|0µM-0h
chr20:36147678..36147711,-	BLCAP	-2,0764	6,8573E-05	9,2808E-02	down	3µM-1h|0µM-0h
chr18:5295692..5295716,-	ZBTB14	6,4952	1,8309E-07	6,4427E-03	up	3µM-1h|0µM-1h
chr19:54041677..54041684,+	ZNF331	6,1251	7,3718E-12	2,5941E-07	up	3µM-3h|0µM-0h
chr19:45971254..45971258,+	FOSB	4,6676	9,2887E-10	1,6343E-05	up	3µM-3h|0µM-0h
chr6:166401410..166401570,-	LINC00473	2,5737	3,1558E-09	3,7017E-05	up	3µM-3h|0µM-0h
chr20:50179375..50179416,-	NFATC2	4,4732	1,5477E-08	1,3616E-04	up	3µM-3h|0µM-0h

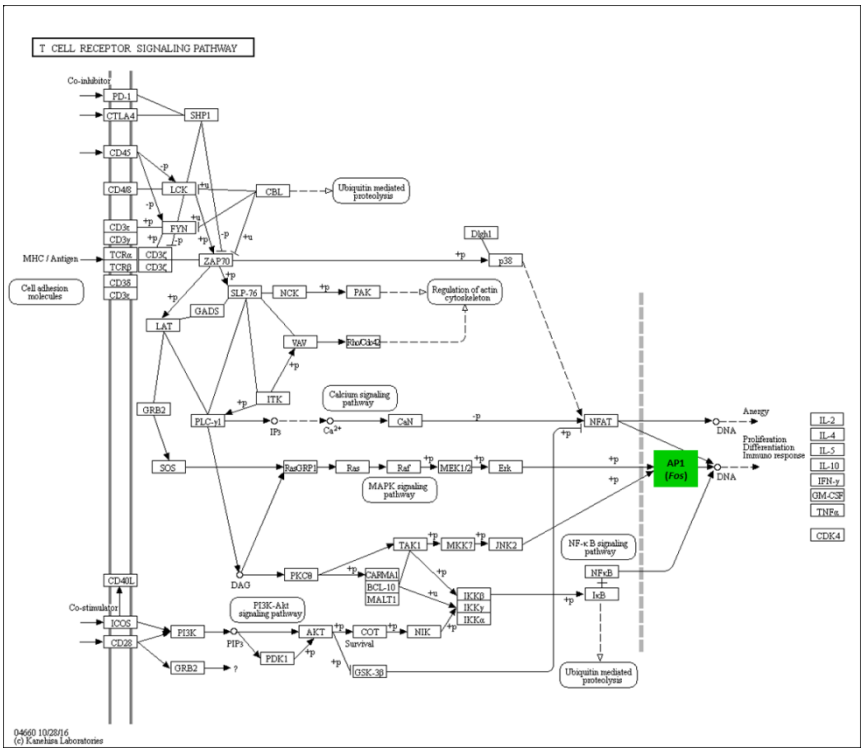
chr17:7747519..7747522,+	KDM6B	3,3496	1,1822E-07	8,3201E-04	up	3µM-3h|0µM-0h
chr12:52445226..52445232,+	NR4A1	3,7482	1,6757E-07	9,8277E-04	up	3µM-3h|0µM-0h
chr3:147127922..147127932,+	ZIC1	2,3751	1,3811E-06	6,9428E-03	up	3µM-3h|0µM-0h
chr5:137801721..137801731,+	EGR1	5,3225	2,0176E-06	8,8746E-03	up	3µM-3h|0µM-0h
chr1:90287518..90287585,+	LRRC8D	2,2585	3,0132E-06	1,1781E-02	up	3µM-3h|0µM-0h
chr13:80912984..80913112,-	SPRY2	1,4926	6,7983E-06	2,3922E-02	up	3µM-3h|0µM-0h
chr19:1203161..1203164,+	STK11	6,3377	1,4147E-05	4,1967E-02	up	3µM-3h|0µM-0h
chr13:102069583..102069593,-	None	3,6817	1,4745E-05	4,1967E-02	up	3µM-3h|0µM-0h
chr10:112257625..112257665,+	DUSP5	2,1480	1,5504E-05	4,1967E-02	up	3µM-3h|0µM-0h
chr12:13349608..13349739,+	EMP1	1,6500	4,2195E-06	9,9027E-02	up	3µM-9h|0µM-0h
chr6:166401410..166401570,-	LINC00473	1,9023	5,6283E-06	9,9027E-02	up	3µM-9h|0µM-0h



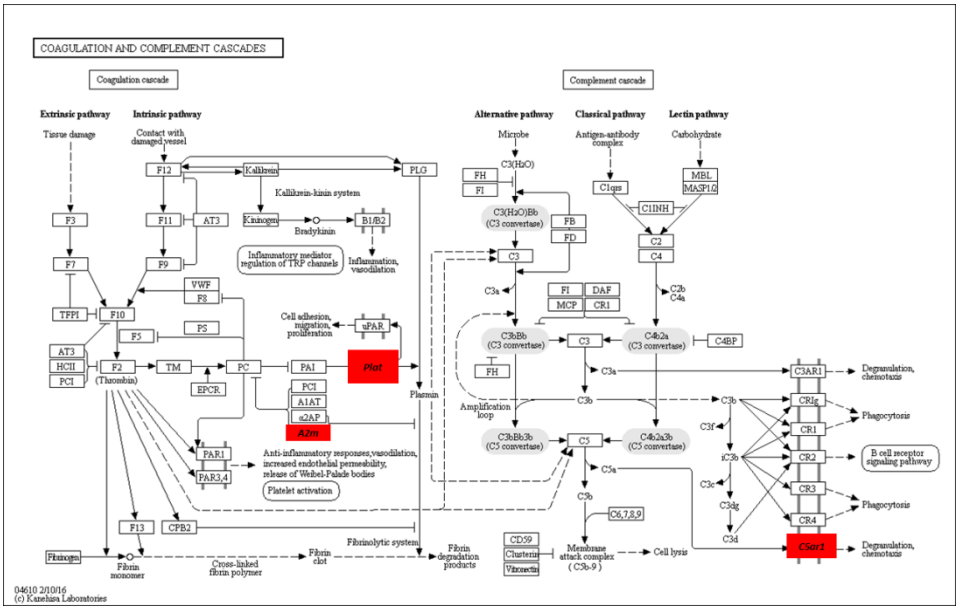
Supplementary Figure S1 – B cell receptor signaling pathway. Genes involved in response to caffeine are highlighted in green, if up-regulated, or red, if down-regulated.



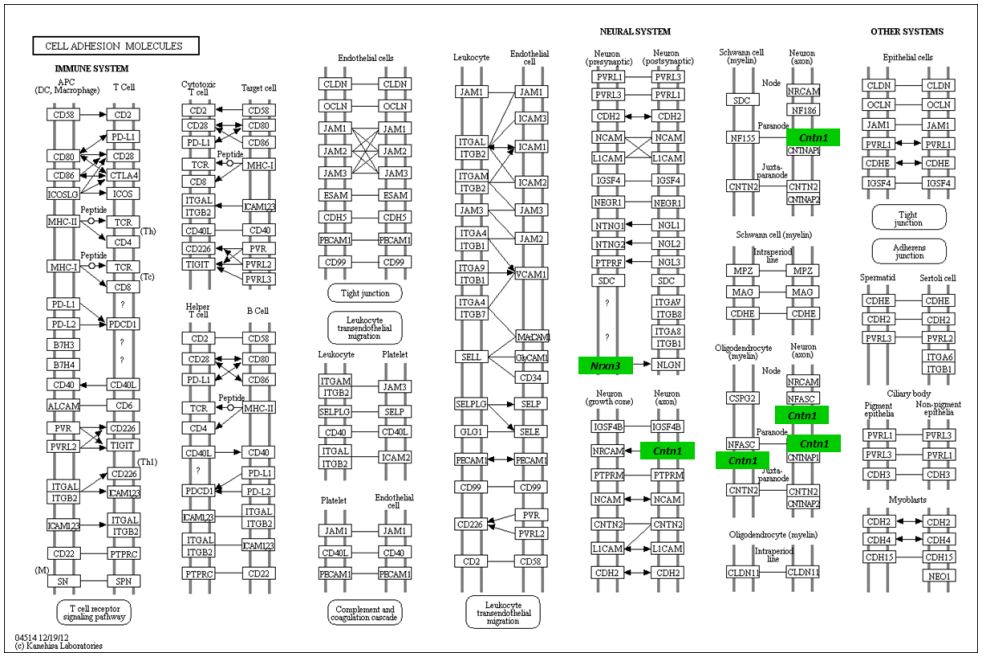
Supplementary Figure S2 – Toll-like receptor signaling pathway. Genes involved in response to caffeine are highlighted in green, if up-regulated, or red, if down-regulated.



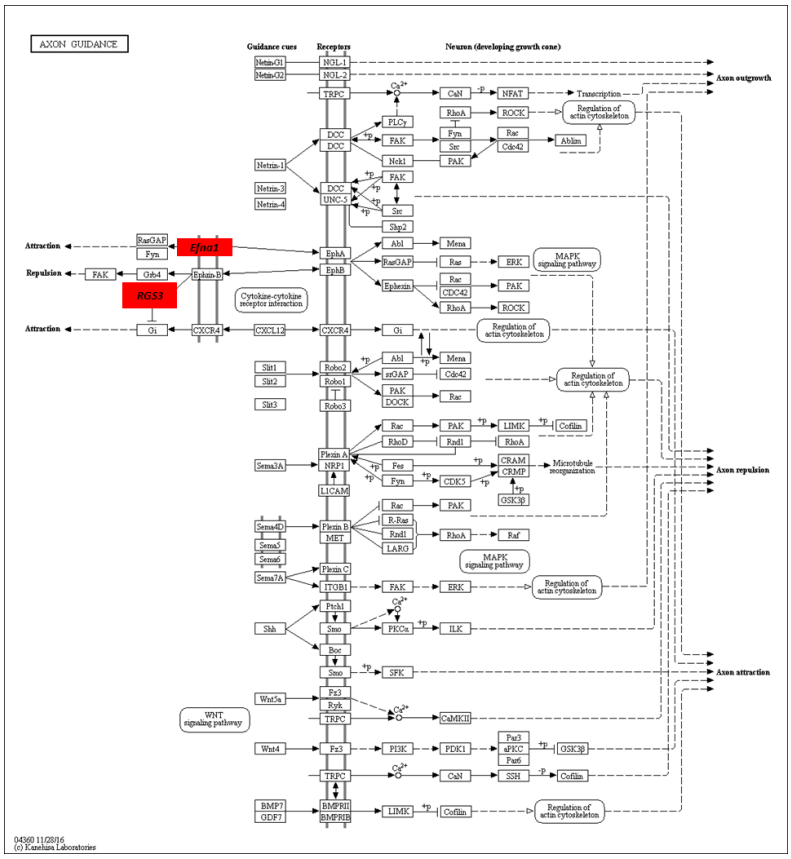
Supplementary Figure S3 – T cell receptor signaling pathway. Genes involved in response to caffeine are highlighted in green, if up-regulated, or red, if down-regulated.



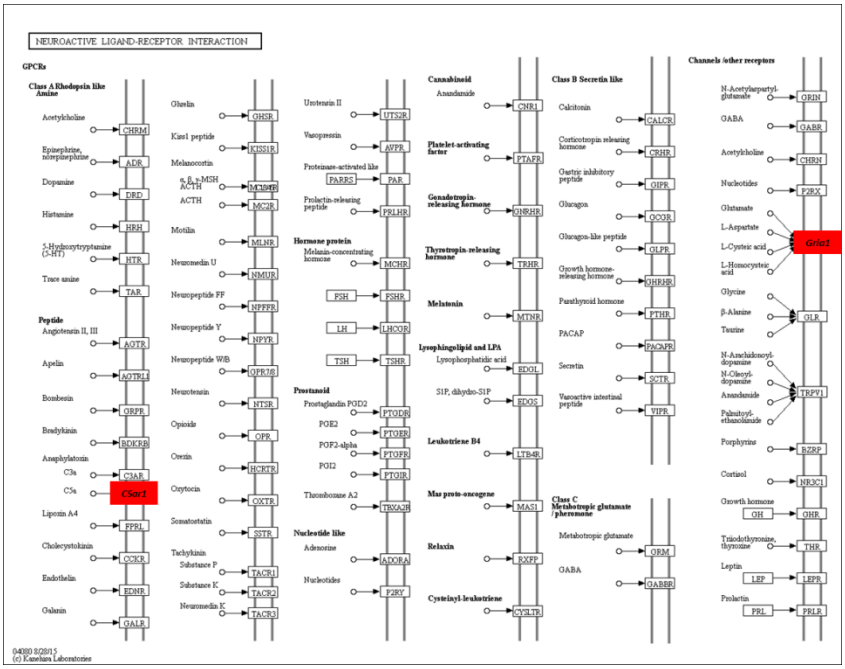
Supplementary Figure S4 – Complement and coagulation cascades pathway. Genes involved in response to caffeine are highlighted in green, if up-regulated, or red, if down-regulated.



Supplementary Figure S5 – Cell adhesion molecules pathway. Genes involved in response to caffeine are highlighted in green, if up-regulated, or red, if down-regulated.



Supplementary Figure S6 – Axon guidance pathway. Genes involved in response to caffeine are highlighted in green, if up-regulated, or red, if down-regulated.



Supplementary Figure S7 – Neuroactive ligand-receptor interaction pathway. Genes involved in response to caffeine are highlighted in green, if up-regulated, or red, if down-regulated.

Supplementary Table 2 – Biological processes perturbed by treatment with 10 µM of caffeine. Gene ontology (GO) analysis results performed in order to understand in which biological processes the caffeine response mediator genes are involved with corresponding statistical information and list of genes related to each process.

ID	Name	P-value	FDR	Number of genes in GO term	Genes in GO term
GO:0022008	neurogenesis	1,05E-05	1,54E-02	20	SOX2, PRRX1, RARA, TNC, MXRA8, CAV1, PTN, EFNA1, EFNA4, NRXN3, RGS6, DKK1, EFHD1
GO:0030182	neuron differentiation	1,72E-05	1,54E-02	18	SOX2, PRRX1, RARA, TNC, CAV1, PTN, EFNA1, EFNA4, NRXN3, RGS6, DKK1, EFHD1, NFIX
GO:0050803	regulation of synapse structure or activity	1,99E-05	1,54E-02	8	VGF, RARA, GRIA1, PTN, RIN1, NRXN3, DKK1, AGT
GO:0031099	regeneration	2,38E-05	1,54E-02	7	SOX2, PRRX1, TNC, CAV1, SULF2, C5AR1
GO:0023056	positive regulation of signaling	5,00E-05	1,93E-02	19	SOX2, CMTM3, PRRX1, RBPMS, RARA, ANGPT1, TRIP6, SOCS3, GRIA1, CAV1, PTN, EFNA1, SULF2
GO:1903598	positive regulation of gap junction assembly	5,32E-05	1,93E-02	2	CAV1, AGT
GO:0048699	generation of neurons	5,60E-05	1,93E-02	18	SOX2, PRRX1, RARA, TNC, CAV1, PTN, EFNA1, EFNA4, NRXN3, RGS6, DKK1, EFHD1, NFIX
GO:0010647	positive regulation of cell communication	5,98E-05	1,93E-02	19	SOX2, CMTM3, PRRX1, RBPMS, RARA, ANGPT1, TRIP6, SOCS3, GRIA1, CAV1, PTN, EFNA1, SULF2
GO:0048167	regulation of synaptic plasticity	8,21E-05	2,36E-02	6	VGF, RARA, GRIA1, PTN, RIN1, AGT
GO:1903596	regulation of gap junction assembly	1,06E-04	2,59E-02	2	CAV1, AGT
GO:0001944	vasculature development	1,29E-04	2,59E-02	11	PRRX1, ANGTP1, SOCS3, CAV1, PTN, EFNA1, NRXN3, AP2B1, AGT, EPC1, C5AR1
GO:0072359	circulatory system development	1,30E-04	2,59E-02	14	PRRX1, RARA, ANGTP1, SOCS3, CAV1, PTN, EFNA1, NRXN3, DKK1, AP2B1, AGT, EPC1, TMEM65
GO:0072358	cardiovascular system development	1,30E-04	2,59E-02	14	PRRX1, RARA, ANGTP1, SOCS3, CAV1, PTN, EFNA1, NRXN3, DKK1, AP2B1, AGT, EPC1, TMEM65
GO:0030030	cell projection organization	2,68E-04	4,93E-02	16	PRRX1, TNC, CAV1, PTN, EFNA1, EFNA4, NRXN3, EFHD1, SPON2, EMP1, MAPK6, HERC1, EMP3

Supplementary Table 3 – Biological processes perturbed by treatment with 3 µM of caffeine. Gene ontology (GO) analysis results performed in order to understand in which biological processes the caffeine response mediator genes are involved with corresponding statistical information and list of genes related to each process.

ID	Name	p-value	FDR	Number of genes in GO term	Genes in GO term
GO:0071277	cellular response to calcium ion	6,08E-04	2,60E-02	2	FOS, FOSB
GO:0009719	response to endogenous stimulus	6,35E-04	2,60E-02	6	TMBIM6, EGR2, EGR3, FOS, FOSB, NPAS4
GO:0021660	rhombomere 3 formation	6,99E-04	2,60E-02	1	EGR2
GO:0021594	rhombomere formation	6,99E-04	2,60E-02	1	EGR2
GO:0099537	trans-synaptic signaling	9,57E-04	2,97E-02	4	EGR2, EGR3, NAT8L, NPAS4
GO:0007268	chemical synaptic transmission	9,57E-04	2,97E-02	4	EGR2, EGR3, NAT8L, NPAS4
GO:0098916	anterograde trans-synaptic signaling	9,57E-04	2,97E-02	4	EGR2, EGR3, NAT8L, NPAS4
GO:0099536	synaptic signaling	1,01E-03	2,97E-02	4	EGR2, EGR3, NAT8L, NPAS4
GO:0007422	peripheral nervous system development	1,38E-03	2,97E-02	2	EGR2, EGR3
GO:0071386	cellular response to corticosterone stimulus	1,40E-03	2,97E-02	1	NPAS4
GO:0035283	central nervous system segmentation	2,09E-03	3,37E-02	1	EGR2
GO:0035284	brain segmentation	2,09E-03	3,37E-02	1	EGR2
GO:0051025	negative regulation of immunoglobulin secretion	2,09E-03	3,37E-02	1	TMBIM6
GO:0097305	response to alcohol	2,36E-03	3,53E-02	3	FOS, FOSB, NPAS4
GO:0010243	response to organonitrogen compound	2,37E-03	3,53E-02	4	TMBIM6, EGR2, FOS, FOSB
GO:0051591	response to cAMP	2,54E-03	3,53E-02	2	FOS, FOSB
GO:0051586	positive regulation of dopamine uptake involved in synaptic transmission	2,79E-03	3,53E-02	1	NAT8L
GO:1903297	regulation of hypoxia-induced intrinsic apoptotic signaling pathway	2,79E-03	3,53E-02	1	TMBIM6
GO:0051592	response to calcium ion	3,37E-03	4,07E-02	2	FOS, FOSB
GO:1901700	response to oxygen-containing compound	3,46E-03	4,07E-02	5	TMBIM6, EGR2, FOS, FOSB, NPAS4
GO:1990144	intrinsic apoptotic signaling pathway in response to hypoxia	4,18E-03	4,52E-02	1	TMBIM6
GO:0001661	conditioned taste aversion	4,18E-03	4,52E-02	1	FOS