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Comparison of RNA extraction by Arpengen kit with RNA extraction by TRIzol and the subsequent use of the kit in a gene expression study

Master´s thesis
30 EAP

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INFOLEHT

RNA eraldamise meetodite võrdlus Arpengeni kiti ja TRIzoli meetodeid kasutades ning Arpengeni kiti kasutamine geeniekspressiooniuuringus

Geeniekspressiooni uuringutes kasutatav RNA peab olema kontaminatidest puhas ning piisavalt intaktne, et saada usaldusväärseid tulemusi. Antud töös on võrreldud membraaniga kolonni kasutatavat Arpengen kitt ja TRIzol-i lahusega RNA eraldamist erinevates koetüüpides, hinnates RNA kvaliteeti spektrofotomeetria, geelelektroforeesi ning RT-qPCR-iga. Lisaks sellele, et selgitati, kas kitt eraldab piisavas koguses ja piisavalt kvaliteetset RNA-d, kasutati seda RNA eraldamiseks hiirte prefrontaal koorest geeniekspressiooni analüüsiks, et uurida, kas *Negr1* geen mõjutab endokannabinoidi süsteemi komponentide ja vere-ajubarjääri molekul claudiin-5 ekspressiooni. Tulemused näitasid, et Arpengen-i kitiga eraldatud RNA on piisavalt kvaliteetne ning kitt on kasutajasõbralikum ja tulemused reprodutseeritavamad võrreldes TRIzoli meetodiga. Geeniekspressiooni uuringu analüüs näitas, et *Negr1*-puudulikkus mõjutab kannabinoidi retseptor 1 ja claudiin-5 ekspressiooni taset vastusena lühiajalisele rasvasele toidule.

Märksõnad: RNA eraldus, RNA kvaliteet, Trizol, Negr1, Endokannabinoidsüsteem

CERCS: T490 Biotehnoloogia; B640 Neuroloogia, neuropsühholoogia, neurofüsioloogia

Comparison of RNA extraction by Arpengen kit with RNA extraction by TRIzol and the subsequent use of the kit in a gene expression study

RNA used in gene expression studies must be free of contaminants and sufficiently intact to obtain reliable results. This work compares the Arpengen kit with a membrane column and the Trizol solution, on tissues that are difficult to process estimating the RNA quality using spectrophotometry, gel electrophoresis and RT-qPCR. After determining that the kit isolates RNA with sufficient quality, is user-friendlier and the result more reproducible than TRIzol extraction, it was used to isolate RNA from the prefrontal cortex of mice for gene expression analysis to investigate whether the *Negr1* gene interacts with selected components of the endocannabinoid system and the blood-brain barrier molecule claudin-5. The results showed that *Negr1*-deficiency affects the expression level of cannabinoid receptors and claudin-5 in response to acute high-fat diet exposure.

Keywords: RNA extraction, RNA quality, Trizol, Negr1, Endocannabinoid system

CERCS: T490 Biotechnology; B640 Neurology, neuropsychology, neurophysiology

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ABBREVIATIONS

2-AG - 2-arachidonylglycerol

BBB - blood-brain barrier

CB1 (*Cnr1*) - cannabinoid receptor 1

CB2 - cannabinoid receptor 2

Cldn5 - claudin 5

CT - cycle threshold

DAGLa (*Dagla*) - diacylglycerol lipase α

ESC - endocannabinoid system

FAAH (*Faah*) - fatty acid amide hydrolase

GWAS - genome wide association studies

HFD - high-fat diet

Hprt – hypoxanthine phosphoribosyltransferase

MGL (*Mgll*) - monoacylglycerol lipase

NEGR1 (*Negr1*) - Neuronal growth regulator 1

PFC - prefrontal cortex

RIN - RNA integrity number

-RT - no reverse transcription

RT - reverse transcribed

RT-qPCR - Reverse transcription quantitative polymerase chain reaction

TBE - Tris-borate-EDTA

INTRODUCTION

Using high quality RNA is essential for the successful application in classical gene expression profiling, like fluorescence-based real time RT-qPCR that has become a standard method for research and molecular diagnostics. High quality sample material is implied to be RNA that is non-degraded or fragmented, free from contamination and of sufficient amount. Working with low-quality RNA may compromise the experimental results of downstream applications which are labour-intensive, time-consuming, and highly expensive. Hence, the analysis of RNA quality prior to processing should be performed in regard to the extraction method.

Currently, there are many methods that can be used to extract pure biomolecules, such as solution-based and column-based protocols. Several studies have identified differences between RNA extraction methods in the quantity and quality of RNA samples, and in the analysis of mRNA and target gene expression. The choice of extraction method can impact the sensitivity and specificity of downstream applications.

This work aims to compare both types of RNA isolation, the Arpengen kit with a membrane column and the TRIzol solution, from various tissues using spectrophotometry, gel electrophoresis and RT-qPCR, hypothesizing that the Arpengen kit will provide higher quality RNA more consistently across tissue types. Second part of this work aims to utilize the kit in extracting RNA for gene expression analysis to study if the effect of neuronal growth regulator 1 (*Negr1*) on the etiology of psychiatric disorders is modulated through changes in the endocannabinoid system (ECS). As both *Negr1* and ECS have a role in similar psychiatric disorders and physiological processes in the brain, it is possible that ECS components are differentially expressed in *Negr1* deficient mice. In addition, since transcriptional changes may not be detectable at baseline, a state of metabolic stress was induced in a group of mice by exposing them to 1 hour of high-fat diet.

This work was carried out in the department of physiology of the University of Tartu.

4. LITERATURE REVIEW

4.1. Gene expression analysis

One of the most important techniques implemented in the research of molecular mechanisms and networks in various cells of different origins is gene expression analysis. It is a well-established method that gives a good insight into the physiological status of the cell (Buckingham and Flaws, 2007). This field has evolved exponentially in the last years as new methods are found that help gain insight into whole transcriptomes of cells, starting from single gene expression to entire libraries of RNA (Romero *et al*.,2014; Buckingham and Flaws, 2007).

With that being said, a lot of the gene expression evaluation depends on the amount and quality of total RNA obtained from cells and tissues. Working with nucleic acids requires very precise conditions and care, as the molecular biology methods are very sensitive and results may vary considerably based on the purity and integrity of the obtained materials (Toni *et al*., 2018; Sellin Jeffries *et al*., 2014; Nouvel *et al*., 2021). So, in order to acquire the most accurate results in downstream applications, certain conditions must be met when working with RNA, especially in material collection, tissue processing and RNA extraction.

4.2. Liquid-based RNA extraction (TRIzol)

Among the different ways of RNA extraction from the starting material of interest, there are key steps that all isolation methods follow - the lysis of tissue and cells, inactivation of ribonucleases, elimination of contaminants, and recovery of total RNA (Buckingham and Flaws, 2007; Tan and Yiap, 2013).

The first step, tissue homogenization, should be done in conditions where RNases are deactivated, such as tissue disruption in liquid nitrogen or using a buffer that deactivates RNases (Nouvel *et al*., 2021). Probably the most widely used chemical compound to disrupt the cells, effectively denature proteins and deactivate nucleases is guanidinium isothiocyanate (commercially available as TRIzol, TriFast, or TRI Reagent) (Chomczynski and Sacchi, 1987; Peirson and Butler, 2007, illustrated on Figure 1). For RNA to be separated from the rest of the cellular material, the mixture should be acidic. With the addition of phenol and chloroform, RNA can be successfully extracted by phase separation, where it remains in the upper aqueous layer. The RNA is later precipitated with isopropanol (Buckingham and Flaws, 2007; Peirson and Butler, 2007; Tan and Yiap, 2013).

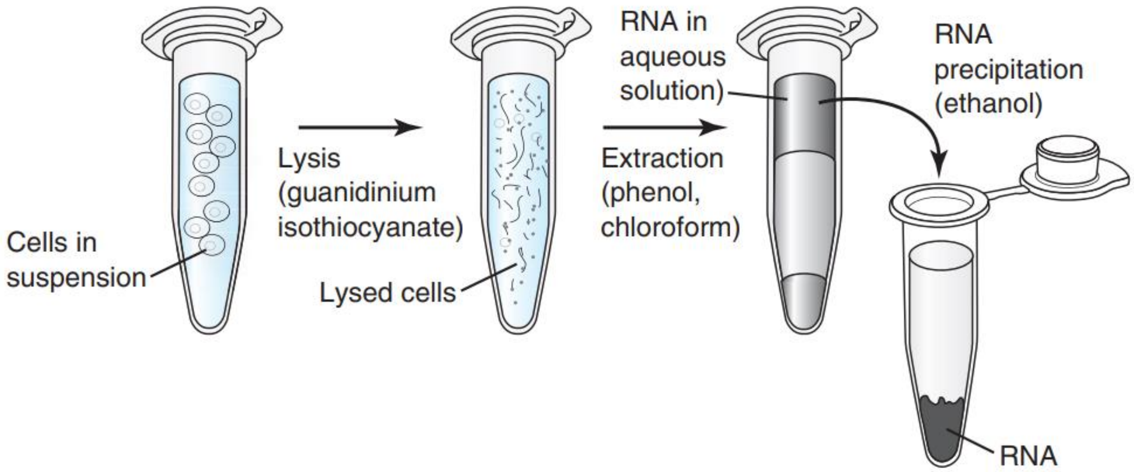

Figure 1. Illustration of liquid-based extraction of RNA. Adapted from Buckingham and Flaws, 2007.

Although considered by some to be the gold standard, liquid-liquid extraction with guanidinium isothiocyanate and phenol has its drawbacks. The process is time-consuming and laborious, which poses a risk of carryover of multiple contaminants, such as guanidinium isothiocyanate and phenol, also proteins and genomic DNA (Peirson and Butler, 2007; Unger *et al*., 2019). Salts and organic compounds have been found to compromise and inhibit future experiments, while the presence of genomic DNA makes the assessment of RNA yield and gene expression levels inaccurate (Bustin and Nolan, 2004; Qiagen, 2018; Buckingham and Flaws, 2007).

Another point to consider is the hazardous nature of the commonly used extraction mixture. Phenol, chloroform and guanidinium isothiocyanate are highly dangerous chemicals that require specialized equipment, waste disposal protocols, and caution when working with.

Additionally, reproducibility issues should be considered. Manual protocol steps (e.g., phase separation, pipetting) introduce variability between users and experiments (Chomczynski and Sacchi, 1987; Schwochow *et al*., 2012).

4.3. Solid-based RNA extraction (Arpengen kit)

Solid phase RNA extraction (Figure 2) requires the same principles in tissue disruption, with strong denaturing buffers for cell lysis and nuclease denaturation, however the purification of RNA relies on the chemical interaction with a solid membrane, most commonly used in centrifuge spin column tubes. The important key points in the solid phase extraction method are that the substrate attaches specifically to the membrane, the matrix with unwanted molecules can be washed away without detachment of the substrate and that the molecule of interest, in this case RNA, can be released from the membrane under certain conditions (Tan and Yiap, 2013; Gjerse *et al.*, 2009). The specific characteristics of the extraction column, such as pore size, surface area, membrane purity and particle size need to be precisely evaluated for the intended application, as not all columns act similarly with different molecule type separation. The most commonly used membrane material in RNA extractions is silica-based (Gjerse *et al.*, 2009; Buckingham and Flaws, 2007).

After phase separation of RNA, it needs to be specifically attached to the column membrane. Interaction with the membrane is facilitated with the addition of ethanol to the solution with RNA and then attached to the column by centrifugal force. Any contaminants present on the membrane are washed away multiple times using a wash buffer, which should be strong enough to wash away unwanted material but also weak enough not to disturb the attached RNA. After washing steps, the sample is eluted from the column membrane by a buffer (Buckingham and Flaws, 2007; Gjerse *et al.*, 2009; Tan and Yiap, 2013).

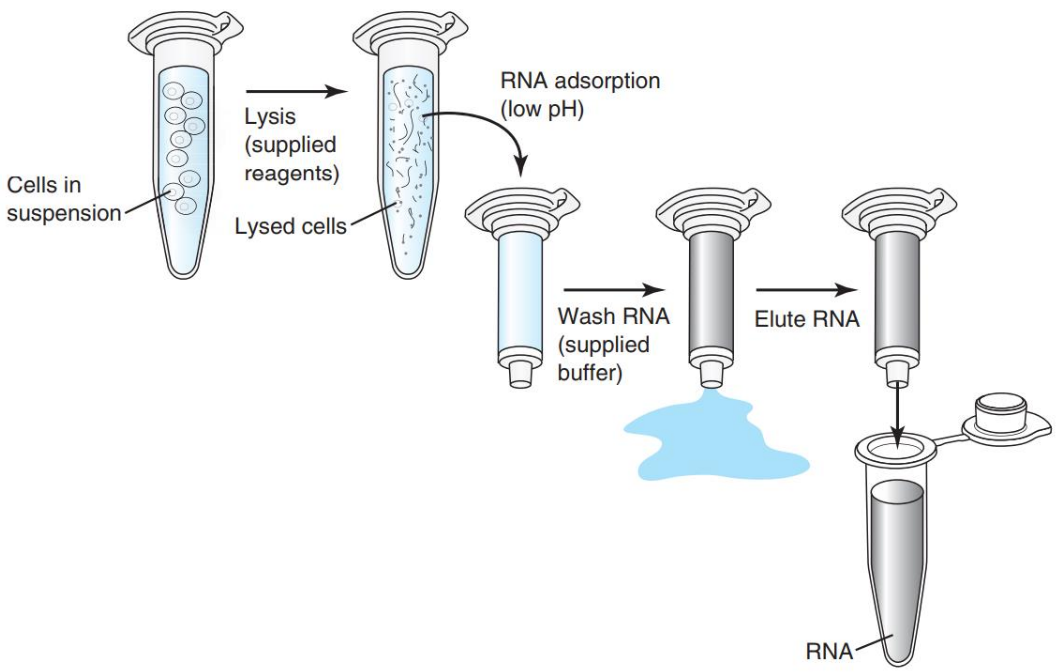

Figure 2. Illustration of membrane-based extraction of RNA. Adapted from Buckingham and Flaws, 2007.

Being the more user-friendly and reliable extraction method, many laboratories start gravitating towards using column-based extraction kits in routine work (Tolosa *et al*., 2007; Escobar and Hunt, 2017; Rump *et al*., 2010). However, there are still some points to consider. Firstly, it is widely reported that solid-phase extraction kits yield lower amounts of total RNA compared to liquid-phase extraction methods (Tesena *et al.*, 2017; Alves *et al.*, 2016; Kanani *et al.*, 2019). This can be attributed to the limited affinity of the column membrane, which is in turn, dependent on the material used in the membrane. Some columns bind very specifically but with a smaller amount, while other columns capture more of the sample but with less specificity, in which case the column will be overloaded and clogged (Gjerse *et al.*, 2009).

4.4. Quality control of the extracted RNA

Manufacturers of different extraction kits and reagents frequently promise the purification of nucleic acids free of contaminants, but in the end, the result is highly dependent on the user and the conditions set for the experiment. There are multiple ways to check whether acquired RNA is sufficiently pure for downstream applications.

The classical optical density measurement of absorbent ratios is frequently used to measure sample concentration and purity with regard to protein and salts. Nucleic acid absorption wavelength is at 260 nm, proteins absorb at 280 nm and higher absorption at 230 nm is indicative of the presence of guanidine thiocyanate, carbohydrates, and peptides (Gallagher and Desjardins, 2006). The analyzer measures the absorption and calculates the ratios of 260/280 and 260/230, with 1.8-2 and >2, respectively, considered pure (Desjardins and Conklin; 2010; Tirabassi, 2024; Buckingham and Flaws, 2007). However, this method can be quite unspecific which makes interpretation questionable. It has been reported that guanidine thiocyanate may absorb differently in different concentrations, absorbing at 230 at lower concentrations and at 260 at very high amounts, resulting in inadequate measure of nucleic acids (Buckingham and Flaws, 2007; Qiagen, 2018). Phenol is also the often-described inhibitor of polymerases and other enzymes resulting in false negatives in amplification assays (Buckingham and Flaws, 2007). Although it is said that the phenol contamination is detectable with a reduced 260/230 ratio, newer methods show that the ratio does not always detect the correct amount of phenol in the sample. Phenol absorbs at 230 nm but also at 270 nm, close to nucleic acid light absorption, resulting in overestimation of concentration, a higher peak at 260 nm with resulting higher ratio of 260/230 (Unger *et al*., 2019). Similarly, the presence of genomic DNA in the RNA sample also overestimates the absorption at 260 nm (Gallagher and Desjardins, 2006). Lastly, absorption ratios may vary a lot with different sample concentrations, where a very low amount of RNA brings the ratio down even if contamination is low, simply because absorption of light at 260 nm is low (Qiagen, 2018).

Another RNA quality check is the assessment of the integrity of the total extracted RNA. The typical way is checking the presence of ribosomal RNA bands by gel electrophoresis (Figure 3) (Edwards, 1974). It is the most abundant type of RNA in the cells so the two subunits, 28S and 18S in eukaryotic cells, appear as two distinct bands of the total RNA sample, visualized by staining with ethidium bromide (Buckingham and Flaws, 2007). In the absence of these bands or heavy smearing, the RNA sample is deemed degraded (Zepeda and Verdonk, 2022).

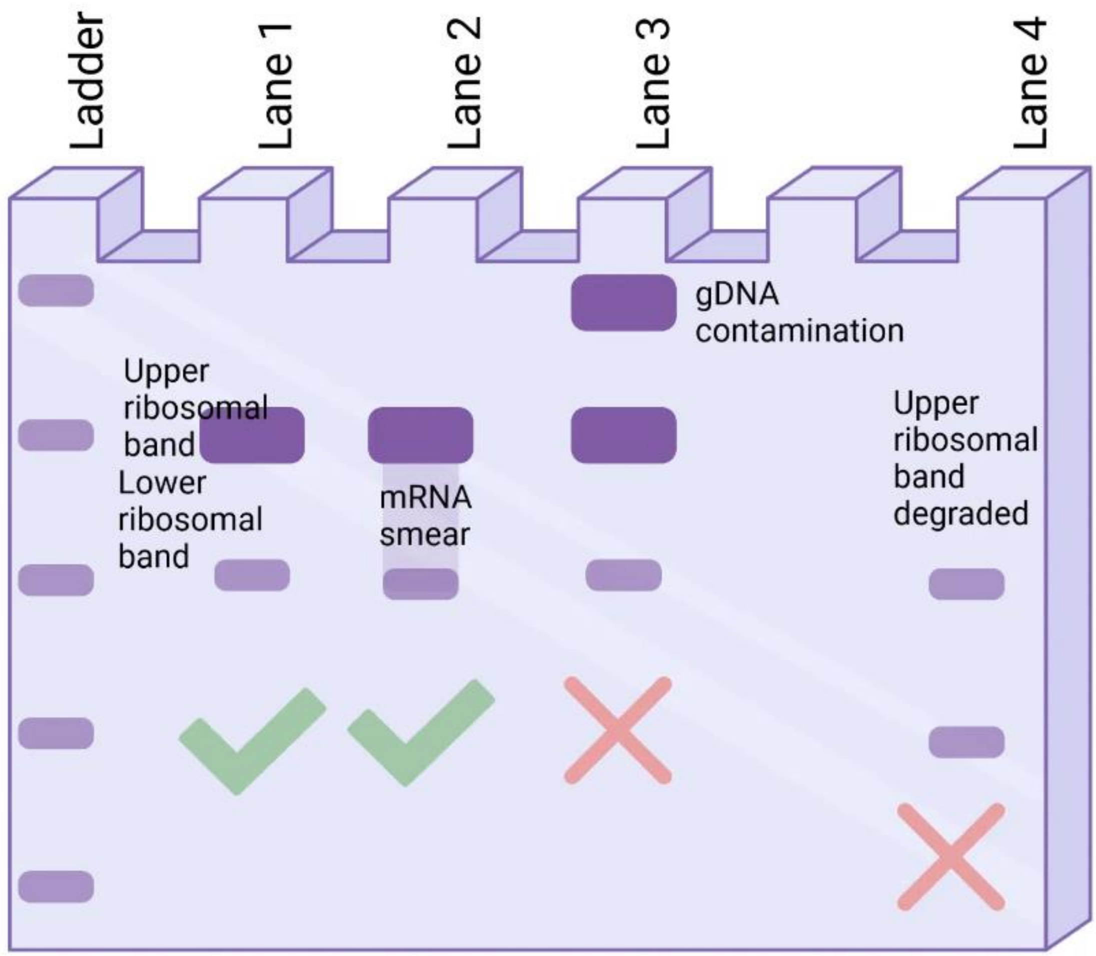

Figure 3. Illustration of analysis of agarose gel with RNA samples. Taken from Tirabassi, 2024.

This way of judging the degradation of RNA is not quantitative and gives only an estimate about the integrity of mRNA. The ribosomal subunits should be at a 2:1 ratio of 28S:18S subunits (Zepeda and Verdonk, 2022; Buckingham and Flaws, 2007). However, on agarose gel, such assessment is only visual and depends on the proficiency of the person doing the experiment, while also consuming a relatively large amount of sample (Bustin and Nolan, 2004).

Newer methods of integrity control use microcapillary electrophoresis with visualization in electropherogram and a virtual gel. The analyzer records fluorescence in the chip channels and calculates an RNA integrity number (RIN) with a ratio of ribosomal subunits while using only a small amount of RNA (as low as 200 pg). This makes RNA integrity assessment easier to analyze, however, being a relatively new method, analytical softwares are currently still being optimized (Imbeaud *et al*., 2005; Schroeder *et al.*, 2006).

4.5. Tissue heterogeneity in RNA extraction

Performing gene expression analysis and transcriptomics on different tissue types from different organisms has its own challenges. Certain mammalian tissues may be problematic and difficult to process varying in size, composition, RNA concentration, RNase activity and matrix effects (Gjerse *et al.*, 2009; Brown et al., 2018). Some tissue types (e.g., adipose, muscle, bone) contain lipids, collagen, or polysaccharides that can co-purify with RNA, affecting purity and downstream applications like RT-qPCR or RNA-seq (Peirson and Butler, 2007; Kanani *et al.*, 2019). Adipose tissue has high fat content but a low cell count, so usually the RNA yield after extraction is suboptimal and many smaller mRNAs may be lost, which causes misleading results in later downstream applications (Cirera, 2013; Nouvel *et al*., 2021). A bigger tissue piece can be used but that means more homogenization buffer and higher fat contents could result in incomplete RNA separation from the rest of the cellular matrix (Brown et al., 2018). Similarly, soft tissues such as brain may also present issues due to higher fat content, but cell count is higher so there are less problems with yield (Brown *et al*., 2018; Rajapaksha *et al.*, 2024). High cell density and rich enzymatic activity are characteristics of liver tissue, while also being one of the tougher tissue types to homogenize due to the presence of high amounts of glycogen, meaning a harsher disruption method may be required, which will compromise some or all RNA integrity (Nouvel *et al.*, 2021; Rajapaksha *et al.*, 2024; Escobar and Hunt, 2017). Abundance of cellular material, especially glycogen, makes precipitation and resuspension in extraction tricky as it co-precipitates with RNA, so additional steps to prevent complications are needed, such as multiple steps of purification (Peirson and Butler, 2007). In case of the on-column extraction, high volumes of co-precipitated RNA clog the membrane and, as mentioned before, more washing steps need to be performed (Escobar and Hunt, 2017; Gjerse *et al.*, 2009). These inconsistencies across tissues present a problem where gene expression analysis results may be skewed.

Many complicated tissue types require specialized approaches to achieve desired RNA quality and yields, so manufacturers offer extraction kits tailored to one tissue type or another (Schwochow *et al.*, 2012; Alves *et al.*, 2016). This however raises the cost for laboratories working with various tissues, meaning more products must be bought (Wex *et al*., 2003). So, there is an increased demand in finding a universal kit which can be used on as many tissue types as needed while being consistent in yielding good amounts of RNA with consistent quality. This may be possible with optimizing a single extraction protocol by modifying, for example, starting material size, lysis buffer amount or adding extra washing steps (Escobar and Hunt, 2017; Boom *et al.*, 1990; Toni *et al.*, 2018; Zeng *et al.*, 2024). This is a more convenient and cheaper way than buying multiple kits and reagents.

4.6. Endocannabinoid system

The endocannabinoid system (ECS) is a central neuromodulatory network responsible for regulation and control of a number of behavioral responses by retrograde inhibition of presynaptic neurotransmitter release. Main components of ECS are the endogenously synthesized ligands endocannabinoids, their receptors and endocannabinoid producing and hydrolyzing enzymes, all of which are widely distributed across the nervous system and peripheral tissues. (Lu, 2015) The two main bioactive lipophilic endocannabinoids in mammals are N-arachidonoyl-ethanolamide (anandamide) and 2-arachidonylglycerol (2-AG). Endocannabinoids are not stored in vesicles but are synthesized and released on demand from membrane lipids (Alger and Kim, 2011). Both 2-AG and anandamide have similar precursors but are synthesized and degraded by different pathways unique to them (Peters and Naneix, 2022). Anandamide is produced by phospholipases D and C and metabolized by fatty acid amide hydrolase (FAAH) while 2-AG is synthesized from diacylglycerol by diacylglycerol lipase, mainly isoform α (DAGLa) in the brain, and metabolized mainly by monoacylglycerol lipase (MGL) (Lu and Mackie, 2015; Peters and Naneix, 2022; Katona and Freund, 2012). The endocannabinoids are agonists of G-protein-coupled cannabinoid receptors 1 and 2 (CB1 and CB2), and the activation of those receptors leads often to blocking the release of vesicles containing neurotransmitters - therefore inhibiting neurotransmitter release from the presynaptic axon terminals (Lu and Mackie, 2015; Peters and Naneix, 2022; Alger and Kim, 2012). CB1 is the most abundant receptor of the ECS found mainly in the central nervous system but also in periphery, while CB2 is a less expressed in the brain and localized mainly in the peripheral tissues (e.g. spleen and immune system) (Navarrete *et al.*, 2020). Endocannabinoids have also different affinities to CB1 and CB2, as studied in the brain, where 2-AG is a much more abundant full agonist with lower binding affinity and anandamide, a partial agonist with higher affinity, meaning 2-AG induces a stronger but shorter response while anandamide induces a weaker signal with longer change in synaptic efficacy (Peters and Naneix, 2022; Katona and Freund, 2012).

4.7. NEGR1

Neuronal growth regulator 1 (NEGR1) is a member of the immunoglobulin superfamily (IgLON) of neural cell adhesion molecules. In the genome wide association studies (GWAS) NEGR1 was shown to have a strong genetic correlation to multiple psychiatric disorders, like bipolar disorder, autism spectrum disorder, schizophrenia, with the strongest link being with depression (Hyde *et al.*, 2016; Wang *et al.*, 2019; Lee *et al.*, 2019). Alterations in the level of *Negr1* in multiple brain regions manifests in various behavioral changes in mice that are associated with psychiatric disorders, such as decline of social interactions, lack of barbering behavior, hyperactivity, memory impairment (Singh *et al.*, 2019; Zhang *et al.* 2025). Several studies have established that one way that *Negr1* dysregulation gives rise to the psychiatric phenotypes is through altered neuronal growth and development, with abnormalities in axonal growth, ventricle size, dendrite length and synapse formation (Pischedda *et al.*, 2016; Singh *et al.*, 2019; Singh *et al*., 2018).
Research to fully understand the underlying mechanisms by which *Negr1* drives the development of psychiatric disorders is still ongoing and one link between the onset of such disorders could be through the ECS. Similarly to *Negr1*, ECS is closely involved in the development of mood disorders, stress regulation, schizophrenia and eating disorders, controlling axonal growth and synaptic patterning (Navarrete *et al.*, 2020; Peters *et al.*, 2022). Wide distribution of ECS in areas or the brain responsible for memory, behavior and mood (e.g. hippocampus and prefrontal cortex) and the dynamic changes in the system during development could be linked with the *Negr1* involvement in neuronal development (Ashton *et al.*, 2017; Di Marzo, 2008).

4.8. Blood-brain barrier

As a part of a pilot study, this work will also touch upon the link with the blood-brain barrier (BBB). The hypothesis being that one way the alterations in the ECS system could influence psychiatric phenotypes of *Negr1*-deficient mice by regulating the BBB function. The ECS components, namely the endocannabinoid receptors, have been found at the neurovascular unit of the BBB and activation of ECS receptors increases the expression of junctional molecules at the BBB, thus regulating its permeability. The selectivity of BBB protects the central nervous system by keeping damaging molecules away, exchanging necessary substances, and ensuring homeostasis- so dysregulation leads to neurological disorders (Hagan *et al.*, 2022). The most abundant type of endothelial tight-junction molecule in the brain is claudin-5. Downregulation of claudin-5 increases BBB permeability, which has been shown to lead to pathogenesis of neurodegenerative and neuropsychiatric disorders (Greene *et al.*, 2019)

5. EXPERIMENTAL PART

5.1. Aims of this thesis

The goal of this methodology study was to evaluate a solid membrane-based RNA extraction kit against a commonly used liquid-based extraction method with TRIzol, more precisely their ability to consistently yield high quality RNA from different tissues. According to previous studies comparing solid- and liquid-phase RNA extractions, there is no concrete answer to which method is better, as results vary with the criteria used to compare them. The general hypothesis was that the Arpengen RNA extraction kit would consistently yield better quality and purer RNA from various tissue types, which is more suitable for downstream applications. After comparison, the kit is used to extract RNA from mice prefrontal cortices to analyze gene expression.

The aims of the thesis are:

1. Compare and evaluate two RNA extraction methods in different tissues by following metrics - total yield, purity, contamination, gene expression
2. Use the kit to extract and measure mRNA expression levels of genes associated in the endocannabinoid system in *Negr1*$^{-/-}$ and *Negr1*$^{+/+}$ (*wt*) mice prefrontal cortices under different diet exposures.

5.2. Methods and materials

5.2.1. Mice

Male and female wild-type (WT) mice and their homozygous *Negr1*-deficient littermates (*Negr1*$^{-/-}$), described previously (Kaare *et al.*, 2021) in F2 background ((129S5/SvEvBrd × C57BL/6N) × (129S5/SvEvBrd × C57BL/6N)), were used in the present study. Mice were group-housed in standard laboratory cages measuring 42.5 (L) × 26.6 (W) × 15.5 (H) cm, with 8 animals per cage in the animal colony, at 22 ± 1 °C under a 12:12 h light/dark cycle (lights off at 19:00 h). A 2 cm layer of aspen bedding (Tapvei, Estonia) and 0.5 l of aspen nesting material (Tapvei, Estonia) were used in each cage and changed every week. Water and food pellets (R70, Lactamin AB, Sweden) were available ad libitum. Breeding and the maintenance of the mice were performed at the animal facility of the Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia. The use of mice was conducted in accordance with the regulations and guidelines approved by the Laboratory Animal Centre at the Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia. All animal procedures were conducted in accordance with the European Communities Directive (2010/63/EU) from the Estonian National Board of Animal Experiments (Kaare *et al.*, 2021).

Two different cohorts of mice were used in this thesis. The first cohort used for the methodology part of this thesis consisted of 6 male *Negr1* $^{+/-}$ mice (a surplus from the breeding).

The second cohort consisted of 31 male *Negr1*$^{-/-}$ mice and their wildtype littermates that were used in previous experiments of currently unpublished studies. Half of the mice of either genotype were on standard diet and half were exposed for 1 h to high-fat/high-sugar diet (HFD). Mice were single-housed for 1 week before this experiment to be able to measure their individual food intake.

High-fat (HF) chow (DIO-45 kJ% fat (lard)) corresponds to the D12451 diet from Ssniff Spezialiäten GmbH (Soest, Germany) and its physiological energetic value is 4.615 kcal/kg. It contains 45 kJ% fat, 20 kJ% proteins, and 35 kJ% carbohydrates. This diet is characterized by high fat content (lard) and high sucrose levels. The caloric value of standard chow (V1534-000 rat/mice universal maintenance diet, autoclavable (10mm) from Ssniff Spezialiäten GmbH, Soest, Germany) corresponds to 3.225 kcal/kg. It contains 9 kJ% fat, 24 kJ% proteins, and 67 kJ% carbohydrates. (Kaare *et al.*, 2021)

5.2.2. Collection of tissues

Mice were decapitated and tissues were dissected by a specialist owning the FELASA-C certificate. Pieces of up to 50 mg of liver and perigonadal fat tissue were cut and flash frozen in liquid nitrogen. Hypothalamus, hippocampus and prefrontal cortices were also dissected from the brains and flash frozen in liquid nitrogen. Tissue tubes were marked and stored in -80 °C.

5.2.3. RNA extraction with TRIzol

Upon taking out of the -80 °C freezer all tissue pieces were held on ice. Brain and fat tissues were homogenized by hand using the micropestle (Eppendorf Micropestle) immediately after the tissues were slightly thawed with the addition of 200 µl of TRIzol reagent (Life technologies). Homogenization continued until there were no visible tissue particles and thereafter, 300 µl of TRIzol was additionally added and the homogenate was incubated for 5 minutes. For liver tissues, due to their density and heterogeneity, homogenization was performed using magnetic beads. 200 µl of TRIzol reagent was added to the slightly thawed tissue and homogenized in the machine. Thereafter, another 300 µl of TRIzol was added and incubated for 5 minutes. After incubation, 100 µl of chloroform was added and shaken vigorously on a vortex machine for 15 seconds, followed by incubation for 2 minutes. Then the tubes were centrifuged for 15 minutes at 12 000 xg at 4 °C. Following the centrifugation, three layers were formed inside the tube: pink, which contains proteins, sugars, and chromosomal DNA; the white lipid layer; and the upper aqueous phase with RNA. The aqueous layer was carefully transferred into a new RNase-free 1.5 ml tube with the addition of isopropanol half the volume of used TRIzol. The solution was carefully mixed by inversion and incubated for 10 minutes. After incubation, the tubes were centrifuged for 10 minutes at 12 000 xg at 4 °C. A visually distinguishable pellet of RNA was formed at the bottom of the tube, its size depending on the tissue size and RNA yield it had, fat tissues yielded no visual pellet. Supernatant was discarded and 1 ml of ice cold 75% ethanol was added followed by centrifugation for 5 or 7 minutes at 7500 or 10000 xg at 4 °C (protocol was adjusted based on the detachment of RNA pellet at the bottom of the tube). Supernatant was removed and the RNA pellet dried as much as possible. RNA was dissolved in 50 µl of nuclease-free water (Solis BioDyne). The concentration of RNA and the 230, 280, 260 nm absorption were measured with NanoDrop (Thermo Fisher Scientific NanoDrop 1000) immediately after extraction. Samples were stored at -80 °C until further use.

5.2.4. RNA extraction using the Arpengen kit

Extractions were performed following the manufacturer's manual. All tissue pieces were held on ice upon taking them out of the -80 °C freezer. Brain and fat tissues were homogenized by hand using the micropestle (Eppendorf Micropestle) immediately after the tissues were slightly thawed with the addition of 300 µl of Lysis buffer. Homogenization was carried out until there were no visible tissue particles and, afterwards, 700 µl of Lysis buffer was added again and the homogenate was incubated for 5 minutes. For liver tissues, homogenization was performed using magnetic beads. 400 µl of Lysis buffer was added to the slightly thawed tissue and homogenized in the machine. Thereafter, another 600 µl of Lysis buffer was added and incubated for 5 minutes. After incubation, 200 µl of chloroform was added and shaken vigorously on a vortex machine for 15 seconds, followed by incubation for 2 minutes. Tubes were centrifuged at 12,000 rpm (13800 xg) for 10 minutes at 4°C. The sample was divided into three layers: the lower blue organic phase, the middle lipid layer and the upper colorless aqueous phase. The RNA is mainly in the aqueous phase. The aqueous phase was transferred to the new RNase-free centrifuge tube. Absolute ethanol (Honeywell) was added to the aqueous phase and mixed carefully by inversion. The volume of absolute ethanol added is 0.5 times the volume of the aqueous phase. The mixed solution with the precipitate was transferred to the spin column, centrifuged at 12,000 rpm (13800 xg) for 30 seconds at 4°C, and the liquid in the tube discarded. 500 µL of Stop Buffer was added to the column, centrifuged at 12,000 rpm (13800 xg) for 30 seconds at 4°C, and the liquid in the tube discarded. 500 µL of Wash Buffer was added to the column, centrifuged at 12,000 rpm (13800 xg) for 30 seconds at 4°C, and the liquid in the tube discarded. The wash step was carried out twice. Then the column was centrifuged at 12,000 rpm (13800 xg) for 2 minutes at 4°C. The spin column was transferred into a new 1.5 mL RNase-free tube, 100µL RElution Buffer was added into the spin column and incubated at room temperature for 1 minute. The columns with tubes were centrifuged at 12,000 rpm (13800 xg) for 1 minute at 4°C, after which the spin column was discarded. The concentration of RNA and the 230, 280, 260 nm absorption was measured with NanoDrop immediately after extraction. Samples were stored at -80 °C until further use.

5.2.5. DNase I digestion

Since the RT-qPCR included two one-exon genes, a decision was made to try the DNase I (Qiagen) reaction on some brain samples to minimize possible genomic DNA contamination in the extracted RNA. There were two ways in which the reaction was performed - on the Arpengen column and in solution. On-column reaction was done after the RNA sample was attached to the column membrane with ethanol followed by one washing step with a wash buffer. 5 µL of DNase I was mixed with 45 µL of RDD buffer (Qiagen) and 50 µL of nuclease free water and added to the column. The reaction was incubated at room temperature for 15 minutes, after which a Stop buffer was added and extraction continued as previously described according to manufacturer's protocol. In-solution digestion took place after the sample was extracted. DNase I and RDD buffer were added directly to the sample and incubated at room temperature for 15 minutes, after which DNase I was inactivated with the addition of 5mM EDTA and 10 minutes of 65℃ heat. Additionally, some samples were inactivated only by heat without the addition of EDTA.

5.2.6. cDNA synthesis

Complementary DNA was synthesized using a reagent kit from Solis BioDyne. The concentration of samples was adjusted to 1000 ng or 200 ng RNA in total, depending on the available RNA yield, and volume brought up with water to 16.5 µL. 2 µL of oligo primers and 1.5 µL of reverse transcriptase enzyme were added to the 0.2 ml tube. The reaction mix was mixed and incubated for 5 minutes at room temperature. Reaction conditions were 55 minutes at 50 °C and inactivation for 5 minutes at 85 °C. cDNA samples were stored in -20 °C.

5.2.7. Agarose gel preparation

The integrity of RNA samples was evaluated by running the samples on 1.5% agarose gel. 3g of pure agarose powder (Bioatlas) was dissolved in 200 µL 0.5x TBE buffer (Bioatlas) by heating the flask in the microwave until the solution was transparent. 12 µL of 10 mg/ml ethidium bromide was added to solution and the liquified gel was poured into the mould with a plate and a comb. The gel solidified after approximately 30 minutes and the comb was removed. The plate with the gel was placed in an electrophoresis tank with 0.5x TBE buffer. 200-400 ng of RNA was mixed with water and 1 µL of 6x loading dye and loaded onto the gel. After all the samples were loaded, 3 µL of 100 bp DNA ladder was loaded onto the gel next to the samples. The samples were run on gel at 150 V for 45-50 minutes, time was corrected according to the separation of DNA ladder bands. The results were viewed under UV light with UVITEC viewer.

5.2.8. Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-qPCR)

The gene expression of RNA samples were evaluated using SYBR green based qPCR method. Primer pairs (TAG Copenhagen) used in the reaction are presented in Table 1. A 3.15 μM primer mix was prepared in advance, containing forward and reverse primers. The experimental master mix was made based on the amount of primers used for a reaction, where for one primer, 9 μL of EvaGreen master mix (Solis BioDyne), 30.1 μL of nuclease free water and 1.6 of diluted cDNA was mixed. Since starting concentration of cDNA was different for some samples, dilutions were calculated according to a final concentration in the reaction well. After dividing the experimental mastermix, 4.3 μL of primer mix was added, making a total of 45 μL, enough for 4 replicates. Each qPCR reaction well contained 10 μL of reaction mix, where a final concentration of cDNA was approximately 2ng/μl. For samples that did not undergo reverse transcription (-RT), about 1 μL of RNA was added with the rest being nuclease-free water. For non-template controls (NTC), cDNA amount was replaced with nuclease-free water. The qPCR was performed in Thermo Fisher Scientific QuantStudio 12k Flex Real-Time machine, using the QuantStudio 12k Flex Software v1.4. Amplification was performed in 40 cycles – primary denaturation for 1 cycle 10 minutes in 95°, the denaturation of the 40 cycles was 95° for 15 seconds, annealing was performed at 60° for 1 minute and extension was performed at 72° for 20 seconds and final extension was at 72° for 5 minutes. The genes analyzed in samples are - cannabinoid receptor 1 (*Cnr1*), claudin 5 (*Cldn5*), diacylglycerol lipase alpha (*Dagla*), monoglyceride lipase (*Mgll*), hypoxanthine phosphoribosyltransferase (*Hprt*), also neuronal growth regulator 1 (*Negr1*) but it was deemed unacceptable as later assessed by melt curves.

Table 1. Primer pairs used in this study. *Hprt* primer pair was designed and validated at the work group in the department of physiology.

Cnr1_mm_Fa	CGTGTTCCACCGCAAAGATAGTC	Clugston *et al.*, 2011
Cnr1_mm_Ra	GTACCTGTCGATGGCCGT	Clugston *et al.*, 2011
Cldn5_mm_Fa	CTCTGCTGGTTCGCCAACA	Liu *et al.*, 2019
Cldn5_mm_Ra	CCCAGCTCGTACTTCTGTGACA	Liu *et al.*, 2019
Faah_mm_Fa	TGAGGAGATGCCCAGCACG	Clugston *et al.*, 2011
Faah_mm_Ra	AGCAAGGTCCAGACTTGGTTATG	Clugston *et al.*, 2011
Faah_mm_Fb	GCAGGTGGGCTGTTCAGT	Dotsey *et al.*, 2017

Faah_mm_Rb	CCAAGCAGGGATCCACAA	Dotsey *et al*., 2017
Dagla_mm_Fa	TGCTGGACGAGGCAAACAA	Clugston *et al*., 2011
Dagla_mm_Ra	CGTAGCATCTCGTGCGAGT	Clugston *et al*., 2011
Mgll_mm_Fa	AAGTTCACCCAGGCGAACTC	Clugston *et al*., 2011
Mgll_mm_Ra	CCACTGGGCTTCCAGTATCTACAA	Clugston *et al*., 2011
Mgll_mm_Fb	GGAACAAGTCGGAGGTTGACC	Manterola *et al*., 2018
Mgll_mm_Rb	CACTCTTGCGACGGCATTC	Manterola *et al*., 2018
Hprt_mm_F	GCAGTACAGCCCCAAAATGG	
Hprt_mm_R	AACAAAGTCTGGCCTGTATCCAA	

5.2.9. Data analysis

For gene expression analysis, the results of amplification cycle thresholds (ct) were first sorted in Microsoft Excel, and $2^{-\Delta\Delta CT}$ was calculated using the ct differences between the target gene and housekeeping gene measured in 4 replicates from the same sample. GraphPad-Prism 10 program was used for analyzing and visualizing the data. Outliers were removed from statistical analysis using the GraphPad-Prism 10 ROUT test. Comparison of metabolic data between groups was performed with a two-way ANOVA test (diet × phenotype), followed by a Fisher's LSD *post-hoc* test. All results are shown in graphs as the mean value ± SEM. Results were considered statistically significant if $p \leq 0.05$.

6. RESULTS

6.1. Comparison of RNA extraction methods

6.1.1. Quality and yield

To compare the Arpengen kit to the TRIzol extraction method, brain, liver and fat tissues weighing between 30-50 mg were obtained from mice and subjected to RNA extraction following provider's protocol. NanoDrop spectrophotometer was used to measure RNA yield and contamination. Starting from the quality of extracted RNA in different tissues, measurements of absorption values showed that nearly all extracted RNA among all tissue types, with the exception of one fat tissue piece extracted with Arpengen kit, is pure from protein contamination, having a 260/280 ratio of more than 1.8 (Table 2.). For the 260/230 ratio, only a few RNA samples from brain tissues reached the pure value of 2 (4 extracted with TRIzol, 1 extracted with Arpengen), with the rest being below the ratio of 2 with high variability, which could indicate some or significant contamination with phenol, salts and carbohydrates. The 260/230 ratios had a much higher variability among tissues, ranging from 0.79 to 2.08 for Arpengen and 0.14 to 2.29 for TRIzol. The average 260/230 ratios vary across tissue types, being higher in brain tissues and lowest in fat tissues, for both TRIzol and Arpengen. The 260/230 ratios appear to be slightly better for RNA samples extracted with Arpengen kit, with the exception of hippocampus, where RNA samples extracted with TRIzol presented near ideal 260/230 ratio values of around 2. Both TRIzol and Arpengen kit are capable of retrieving protein free RNA but with some extraction buffer residues.

The total RNA yield across hippocampus, liver and hypothalamus samples is consistent with previous studies, where the yield is significantly less for samples extracted by spin column kits. For brain samples, the difference is less than 0.5-fold, while RNA extracted from the liver tissue by TRIzol yielded more than 3 times the RNA compared to extractions with the Arpengen kit. On the other hand, RNA yield from fat tissues was almost 3 times higher when extracting with the Arpengen kit compared to TRIzol. Another thing to note is the variability of the yield between different tissue types using the two extraction methods; TRIzol results in a more consistent extraction of similar amounts of RNA across tissues, with the exception of liver.

Table 2. Quality and yield comparison for RNA extracted by TRIzol reagent versus Arpengen kit

260/280 (protein contamination)			260/230 (phenol, salts, carbohydrates)			Yield (ng/mg)		
Tissue	**TRIzol**	**Arpengen**	**Tissue**	**TRIzol**	**Arpengen**	**Tissue**	**TRIzol**	**Arpengen**
hippocampus	1.97	1.91	hippocampus	2.00	0.79	hippocampus	799.8	273.6
n=6	1.96	2.09	n=6	1.98	1.66	n=6	800.9	593.8
	1.90	2.05		2.29	1.78		700.9	442.5
mean	1.94	2.02	mean	2.09	1.41		767.2	436.6
hypothalamus	2.00	2.09	hypothalamus	0.66	2.08	hypothalamus	752.4	689.9
n=6	1.93	1.95	n=6	2.04	1.54	n=6	738.5	263.9
	1.94	2.06		2.02	1.74		884.0	262.4
mean	1.96	2.03	mean	1.57	1.79	mean	791.6	405.4
liver	2.01	2.04	liver	1.57	0.86	liver	4755.7	1152.9
n=6	2.01	2.04	n=6	1.20	1.52	n=6	4273.3	1467.2
	2.01	2.05		1.21	1.70		4842.0	1485.2
mean	2.01	2.04	mean	1.33	1.36	mean	4623.7	1368.4
fat	1.96	1.97	fat	0.14	1.60	fat	25.6	111.3
n=6	1.82	1.69	n=6	1.72	0.96	n=6	26.4	92.9
	1.81	1.80		1.40	1.01		34.1	50.9
mean	1.86	1.82	mean	1.09	1.19	mean	28.7	85.0

6.1.2. RNA integrity and genomic DNA contamination

Next, to assess whether the extracted RNA is sufficiently intact while also identifying genomic DNA presence, the RNA samples were run by gel electrophoresis. For hypothalamic tissues, all RNA samples had the expected distinct 2 bands of ribosomal subunits with faint bands of mRNA in the background. With this method, numerical values for ribosomal subunit ratios cannot be given, but visually, the 28S band should be more prominent than 18S, which is better identifiable in samples extracted with Arpengen kit. In addition, all samples extracted with TRIzol exhibit a distinct band of less than 100 bp, which could be degraded RNA material. The distinct band of high molecular weight material near the wells is the genomic DNA. All hypothalamic RNA samples, irrespective of the extraction method, have potential genomic DNA contamination based on this method. In this particular gel picture (Figure 4.), there are also samples where DNaseI digestion was performed, in which any bands visible above 3000 bp are removed but some genomic DNA at the wells is still visible.

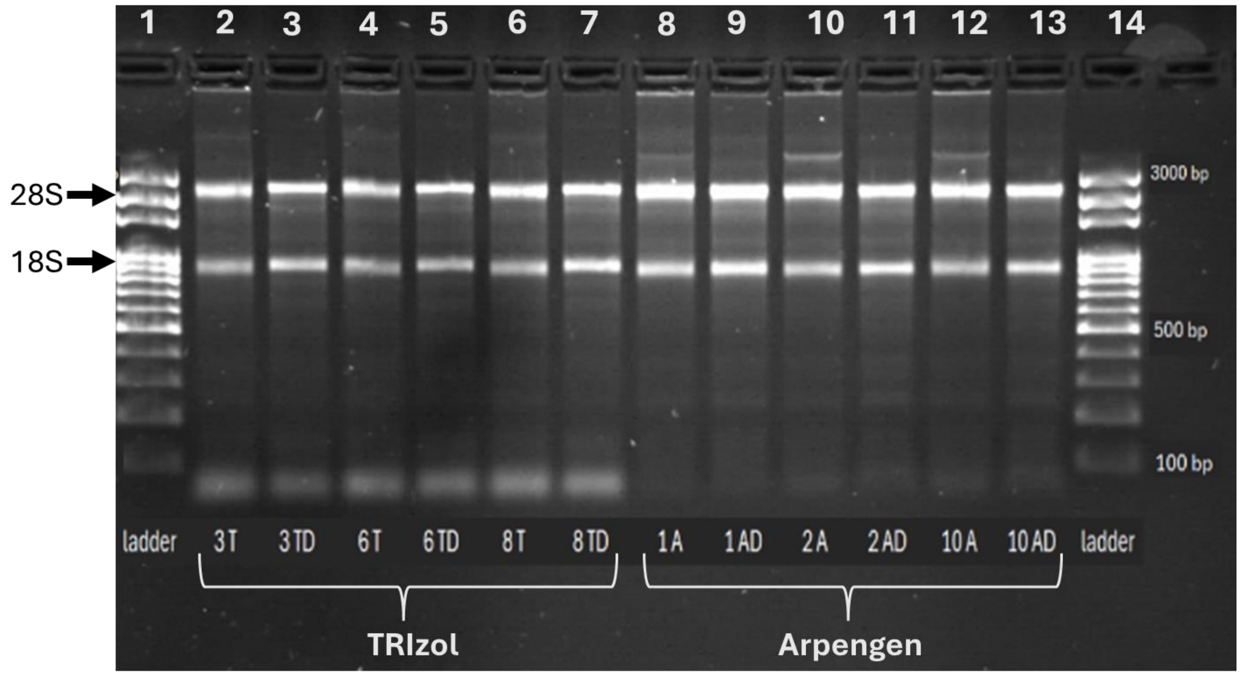

Figure 4. 1.5% agarose gel image with 400 ng total RNA samples extracted from hypothalamus with TRIzol (wells 2-7) and Arpengen kit (wells 8-13). DNaseI treated samples for TRIzol are in wells 3, 5, 7 and for Arpengen in 9, 11, 13. 100 bp DNA ladder is displayed in wells 1 and 14.

Next, liver and hippocampal RNA samples were analyzed. Similarly to before, two distinct bands are visible in all samples, with all TRIzol-extracted samples having visible low molecular weight RNA material. Both TRIzol and Arpengen kit extractions for liver samples exhibit two bands right above the 28S subunit. Liver samples extracted with TRIzol have much heavier smearing of the bands compared to other samples, however that could be attributed to the high RNA concentration, making pipetting 400 ng of sample much more difficult. Again, while being difficult to distinguish, all samples show a 28S ribosomal subunit more fluorescent compared to the smaller counterpart, indicating an acceptable ratio. All liver and hippocampic samples irrespective of extraction method show potential genomic DNA contamination, with a visible band near the wells.

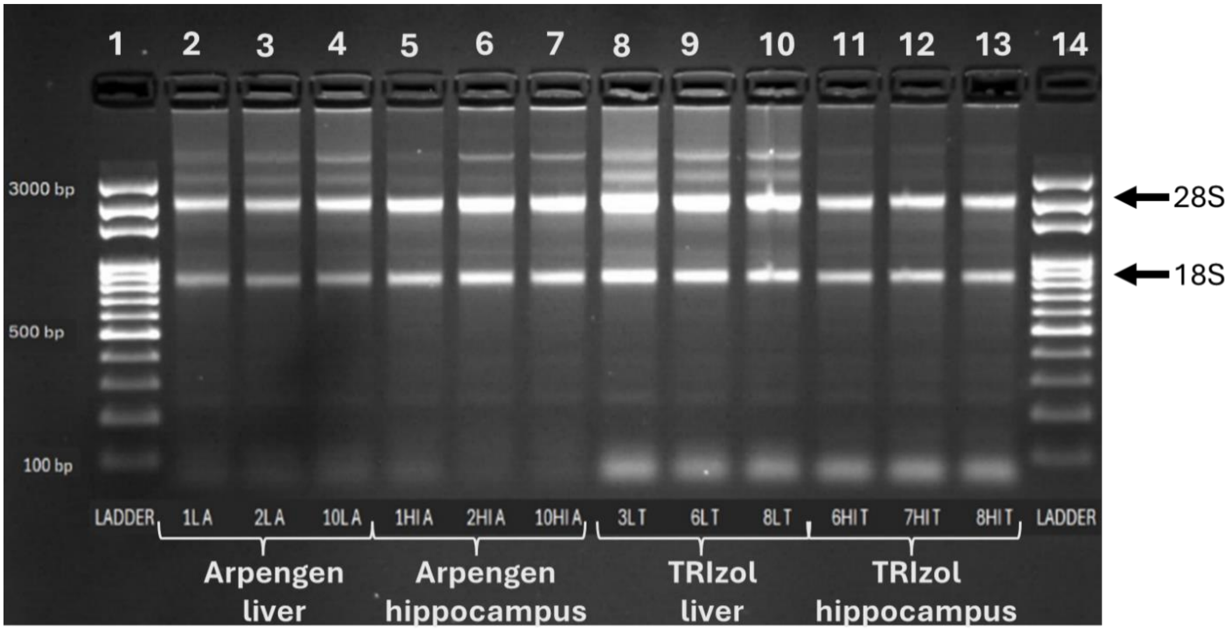

Figure 5. 1.5% agarose gel image with 400 ng total RNA samples extracted from liver and hippocampus with TRIzol (wells 8-13) and Arpengen kit (wells 2-7). 100 bp DNA ladder is displayed in wells 1 and 14.

Lastly, gel electrophoresis of fat tissues was performed using less material as the total amount of extracted RNA was much less compared to other tissues, so the bands appear lighter in comparison, however the ribosomal bands are visible and intact in all samples. The difference is in the amount of genomic DNA in the samples, where TRIzol extracted samples have a highly distinct band near the wells while Arpengen kit extracted samples do not have a clearly distinguishable high molecular weight band. Consistently with previous tissue types, TRIzol-extracted fat tissues exhibit a low molecular weight band under 100 bp, while Arpengen samples do not.

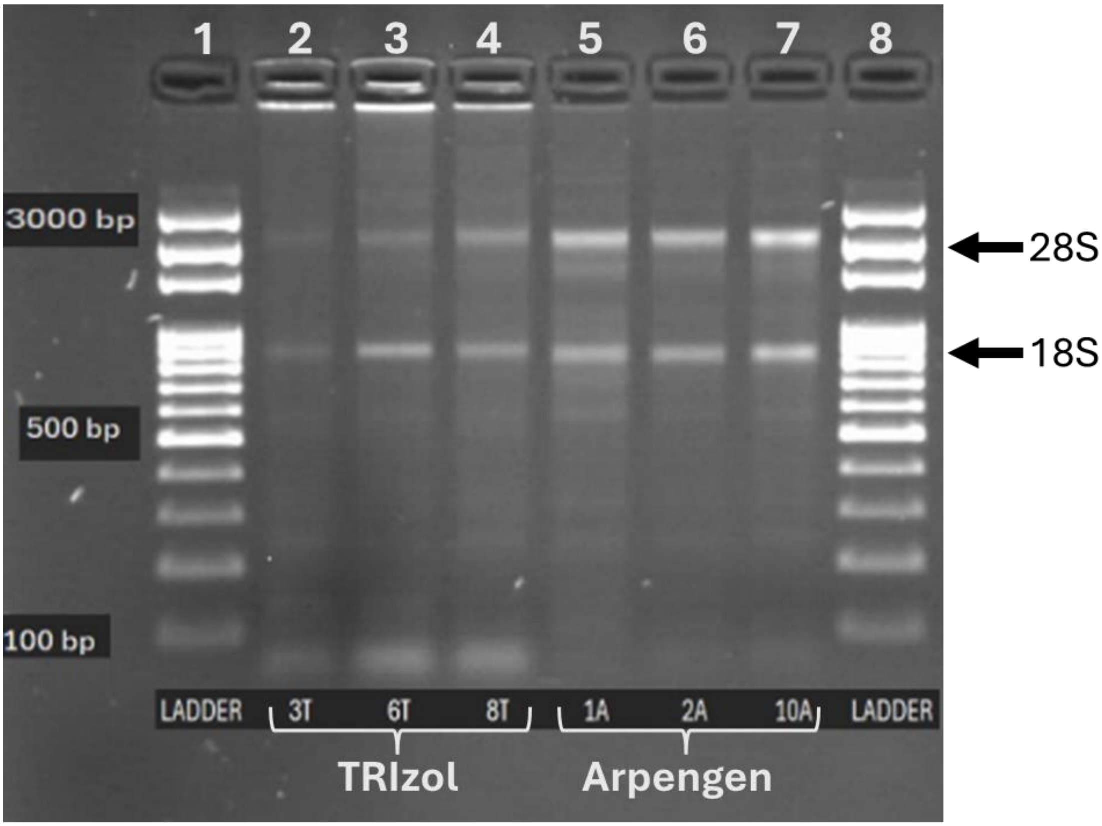

Figure 6.1.5% agarose gel image with 200 ng total RNA samples extracted from fat tissue with TRIzol (wells 2-4) and Arpengen kit (wells 5-7). 100 bp DNA ladder is displayed in wells 1 and 8.

Overall, both extraction methods are capable of extracting sufficiently intact RNA, where two ribosomal bands are visible, but the very distinct low molecular weight band that TRIzol extracted samples exhibit may be indicative that this extraction method degrades some of the RNA. This method of integrity control, however, is not sensitive enough to give a proper comparison of integrity. Additionally, according to the agarose gel results, all samples but fat tissues extracted with Arpengen have genomic DNA contamination. The amount of the DNA present, however, remains unknown.

6.1.3. Gene expression analysis

Another method to evaluate the quality of extracted RNA and the presence of genomic DNA is RT-qPCR. Multiple primers were used in different tissue types with some differences of the target-genes based on their expected expression in the target tissues. For brain tissues, two one-exon and two multi-exon genes were used, for fat, two one-exon and three multi-exon genes and for the liver, one one-exon and three multi-exon genes were used. The choices were made after checking whether the genes are expressed in according tissues. Primers designed for one-exon genes are designed based on that one exon, meaning the amplification may occur on complementary DNA from the mRNA but also genomic DNA. Primers for multi-exon genes were made to span exon-junctions, leaving introns out to avoid unnecessary amplification from genomic DNA.

Before any tests, the course of action for this methodology study was determined based on RT-qPCR results data from the preliminary experiment with 4 samples from mouse hippocampus: 2 of these RNA samples extracted with TRIzol and 2 with the Arpengen kit (Table 3.). Results showed a noticeable difference in the quality between the two extraction methods with Arpengen extracted samples having earlier cycle threshold (ct) values for both the gene of interest and a housekeeping gene. Ct values are used to quantify gene expression of genes of interest and housekeeping genes, reflecting the amount of starting material, with lower ct meaning more template and higher ct the opposite. *Cnr1* being one-exon gene, the amplification happens even in RNA control samples with no reverse transcriptase (-RT) for both extraction methods: However in TRIzol samples, the ct comes earlier in -RT samples than the cDNA samples, whereas in Arpengen samples, with one of the samples having the ct difference between cDNA and -RT samples of over 5 cycles, which is considered to not affect evaluation. Ct difference for the housekeeping gene actin β (*Actb*) expression was present in all samples, however due to the ct-s in the RNA control samples being less than 35, this housekeeping gene was considered suboptimal for further studies and left out.

Table 3. RT-qPCR results for RNA extracted by TRIzol reagent versus Arpengen kit. Ct values are shown for each replicate in every sample. Replicates marked as n/a showed no amplification. cDNA ct values are from complementary DNA samples and RNA control ct values are from samples that did not undergo reverse transcription.

Extraction method		*Cnr1*		*Actb*	
	Hippocampus	cDNA, ct	RNA control, ct	cDNA, ct	RNA control, ct
Trizol	Nr. 1	33.8	31.1	22.7	29.3
	(4 replicates)	33.8	31.4	22.6	29.4
		33.1	30.7	22.8	28.8
		n/a	30.5	22.6	28.4
	Nr. 2	n/a	34.5	23.2	32.9
	(4 replicates)	36.9	33.4	23.2	31.9
		37.5	33.8	23.2	32.1
		38.6	34.5	23.2	33
Arpengen	Nr. 3	24.8	31.4	19.4	29.4
	(4 replicates)	24.9	31.5	19.4	29.6
		24.8	31.3	19.4	29.8
		25.1	31.4	19.4	29.7
	Nr. 4	26.5	29.7	20.4	28.3
	(4 replicates)	26.5	29.4	20.4	28.3
		26.5	29.6	20.4	28.2
		26.6	29.3	20.4	28.8

The RT-qPCR results for the next steps of the methodology part of the given study were unexpectedly different from results shown in the preliminary tests. Based on the results from one-exon genes (*Cnr1* and *Cldn5*), almost all Arpengen extracted samples across tissue types, with exception of one fat tissue, seem to have genomic DNA contamination. The ct values of their RNA control samples which were not treated with reverse transcriptase (-RT) are all under 35 cycles and can thus be considered as detectable signals (Table 4.). Generally, the criteria for assuming genomic DNA contamination is if the ct in RNA control sample is below 35 and if there is under 5 cycle difference between cDNA and -RT RNA control of the same sample.

In Arpengen kit-extracted brain and liver tissues, interestingly, the ct-s of -RT samples are lower than the ct-s of cDNA (RT) samples, showing higher amplification in RNA control samples. Also, the difference between ct values of cDNA and RNA control of the same sample (ΔCT) is less than 5 in Arpengen kit-extracted tissues. Same cannot be said for TRIzol extracted samples. For *Cnr1*, all brain samples and one fat sample show sufficient genomic DNA elimination, as the ct differences between RT and -RT samples are more than 5 cycles, however most -RT samples in the brain have amplification starting earlier than 35 cycles, which is not ideal. For *Cldn5*, most tissue samples had insufficient genomic DNA elimination as most samples had a ct difference between RT and -RT of under 5 cycles and also many -RT samples had amplification under the 35th cycle. Results for multi-exon genes in brain and fat samples show that the primers designed for exon-junction work as intended in case of both extraction methods, with almost no amplification in -RT samples. It should also be mentioned that melt curves in -RT samples of over 36 ct were unspecific, meaning whatever amplification signal appeared was not specific to the target but may have been the result of primer dimers.

Liver was the most problematic tissue type to work with, as it also appears in the final RT-qPCR results. Arpengen kit extracted liver samples showed similar results for one-exon gene as in the brain samples, with earlier amplification in the -RT samples than RT samples, indicating insufficient gDNA removal. The chosen one-exon gene for liver, *Cldn5*, did not work in the Trizol extracted liver samples, with no amplification in RT samples and some amplification in -RT samples. Multi-exon genes presented different issues in both extraction methods, the housekeeping gene *Hprt* worked as intended. In Arpengen extracted livers, *Faah* and *Mgll* in RT samples had mostly sufficient amplification, however both genes were amplified in -RT at cycles lower than 35 with mostly unspecific melt curves, which could be detrimental to accurate gene expression analysis. In TRIzol extracted liver samples *Mgll* and *Hprt* had specific amplification in RT samples and no amplification in -RT samples, while Faah was amplified in only one RT sample. Another thing to note, is that qPCR on liver samples was performed using different primer pairs for *Faah* and *Mgll* than in fat tissues. Different primers were chosen for fat samples after reviewing the results in liver samples.

Table 4. RT-qPCR results for RNA extracted by TRIzol reagent versus Arpengen kit. Values are shown as average ct-s of replicates of the same sample. ΔCT shows difference in cycles between cDNA and RNA control. Samples having - indicate unspecific amplification where the ct difference (ΔCT) could not be calculated. RT - reverse transcribed cDNAs samples, -RT - negative control samples.

	Cnr1			*Cldn5*			*DaglA*			*Faah*			*Mgll*			*Hprt*		
TRIzol	RT	-RT	ΔCT	RT	-RT	ΔCT	RT	-RT	ΔCT	RT	-RT	ΔCT	RT	-RT	ΔCT	RT	-RT	ΔCT
Hippocampus	28.98	36.90	7.93	32.78	36.80	4.03	29.43	>40	10.58							24.98	37.80	12.83
	27.78	34.68	6.90	30.38	32.70	2.33	28.75	39.10	10.35							24.10	39.80	15.70
	29.43	34.55	5.13	32.30	33.25	0.95	29.80	>40	10.20							24.98	>40	15.03
Hypothalamus	28.45	33.93	5.48	30.95	34.10	3.15	30.58	38.10	7.53							24.03	>40	15.98
	28.13	34.63	6.50	30.00	35.13	5.13	28.60	>40	11.40							23.73	39.80	16.08
	28.03	34.38	6.35	30.20	35.03	4.83	31.00	>40	9.00							21.50	38.40	16.90
Fat	31.00	36.40	5.40	31.00	34.10	3.10				31.43	>40	8.58	24.73	>40	15.28	25.10	>40	14.90
	31.73	36.23	4.50	30.35	34.25	3.90				31.28	34.60	3.33	23.60	38.80	15.20	25.18	>40	14.83
	32.40	35.60	3.20	31.18	35.87	4.69				32.10	>40	7.90	24.63	>40	15.38	25.58	>40	14.43
Liver				>40	35.06	4.94				>40	>40	-	33.39	>40	6.61	29.14	>40	10.86
				>40	34.98	5.03				34.77	>40	5.23	33.57	>40	6.43	28.63	>40	11.38
				>40	38.08	1.93				>40	>40	-	33.56	>40	6.44	29.01	>40	10.99

	Cnr1			*Cldn5*			*DaglA*			*Faah*			*Mgll*			*Hprt*		
Arpengen	RT	-RT	ΔCT	RT	-RT	ΔCT	RT	-RT	ΔCT	RT	-RT	ΔCT	RT	-RT	ΔCT	RT	-RT	ΔCT
Hippocampus	29.50	29.55	0.05	30.98	30.38	0.60	31.50	39.27	7.77							25.30	39.50	14.20
	29.05	30.95	1.90	30.55	30.68	0.13	30.28	>40	9.73							25.10	>40	14.90
	28.25	27.00	1.25	29.65	26.70	2.95	30.38	37.17	6.79							25.38	>40	14.63
Hypothalamus	29.35	28.23	1.13	30.65	29.63	1.03	32.03	38.80	6.78							24.70	>40	15.30
	27.85	27.35	0.50	29.43	28.63	0.80	28.20	35.33	7.13							23.68	38.98	15.30
	29.00	28.35	0.65	30.25	29.58	0.68	32.20	38.30	6.10							21.75	37.57	15.82
Fat	30.50	31.48	0.98	28.85	31.60	2.75				31.03	34.68	3.65	25.35	>40	14.65	25.93	>40	14.08
	31.03	33.30	2.28	30.20	34.43	4.23				32.70	37.30	4.60	26.15	>40	13.85	26.48	>40	13.53
	32.98	35.43	2.45	31.28	35.05	3.78				33.40	35.20	1.80	26.05	>40	13.95	26.28	>40	13.73
Liver				33.05	30.90	2.15				36.58	-	-	33.45	-	-	29.65	>40	10.35
				32.29	29.05	3.24				33.32	29.82	3.50	32.16	-	-	29.25	39.95	10.71
				31.33	28.05	3.28				32.44	29.41	3.03	34.81	-	-	28.96	>40	11.04

The RT-qPCR results contradict the gel photos shown before (Figures 4-6) concerning the presence of genomic DNA. Highly fluorescent high molecular weight bands indicative of genomic DNA were visible in all of the extracted samples with both methods with exception of fat tissues extracted with Arpengen kit. Yet, ample amplification in -RT RNA control samples indicative of genomic DNA is prevalent in all Arpengen extracted tissues but not the TRIzol-extracted samples in the RT-qPCR results (Table 4). It was expected based on RNA gel results that all samples would have amplification in -RT RNA control samples with one-exon genes.

6.1.4. Validation of primers

Alongside the comparison of two extraction methods, primers for gene expression analysis of the endocannabinoid system were evaluated for further studies looking at the melt curves of amplicons. One-exon genes were specifically amplified, although DNase treatment of the samples should be considered to avoid amplification from the genomic DNA that could obscure the results. The protocol for that, however, could not be perfected in this study, amplification in RT-qPCR was subdued in all samples where DNaseI treatment was performed. *Dagla* and *Hprt* primers had specific amplification and are exon-spanning and, additionally, no amplification in -RT samples was validated in the current work. The first primer pairs used for *Faah* and *Mgll* in the liver samples had either none or only slight amplification and -RT exhibited high non-specific fluorescent signals (examples of melt curves exhibiting unspecific amplification in additional materials Figure 10.). Additionally, the first *Faah* primer pair used was not spanning exons, which would explain the high amplification rate in Arpengen extracted liver samples. Therefore, a different set of *Faah* and *Mgll* primers were used in fat samples, and must be used for further studies, as it have better stability and properly span exon-exon junctions.

6.2. The gene expression study of ECS with the kit

One of the aims of this thesis was to measure the expression of genes of interest involved in the endocannabinoid system (ECS) and the blood-brain barrier in the prefrontal cortex (PFC) of male *Negr1*$^{-/-}$ and *wt* mice. Before tissue collection, 7 *Negr1*$^{-/-}$ and 8 *wt* mice were exposed to 1h of high-fat diet, other 8 *Negr1*$^{-/-}$ and 8 *wt* mice remained on a standard chow diet. The expression of *Faah* and *Dagla* could not be evaluated in this study as none of the PFC samples showed stable amplification (data not shown). Two genes belonging to the ECS were analyzed - *Cnr1* and *Mgll*, and one gene relevant to the blood-brain barrier - *Cldn5*. Housekeeping gene *Hprt* was used for normalization.

RT-qPCR results analyzed by 2-way ANOVA showed a significant effect of diet ($F (1, 26) = 5.2$, $p = 0.031$) on the gene expression of one of the main endocannabinoid receptors *Cnr1* in *Negr1*$^{-/-}$ and *wt* mice PFC-s. Genotype ($F (1, 26) = 1.03$, $p = 0.32$) and the interaction of diet with genotype ($F (1, 26) = 3.88$, $p = 0.06$) had no significant effect on the *Cnr1* gene expression. *Post hoc* tests of the 2-way ANOVA revealed a highly significant difference ($p = 0.0058$) in Cnr1 expression levels in *wt* mice with due to 1h high-fat diet exposure. *Wt* mice on a standard chow diet had a much higher gene expression or *Cnr1* compared to *wt* mice on a high-fat diet (Figure 7).

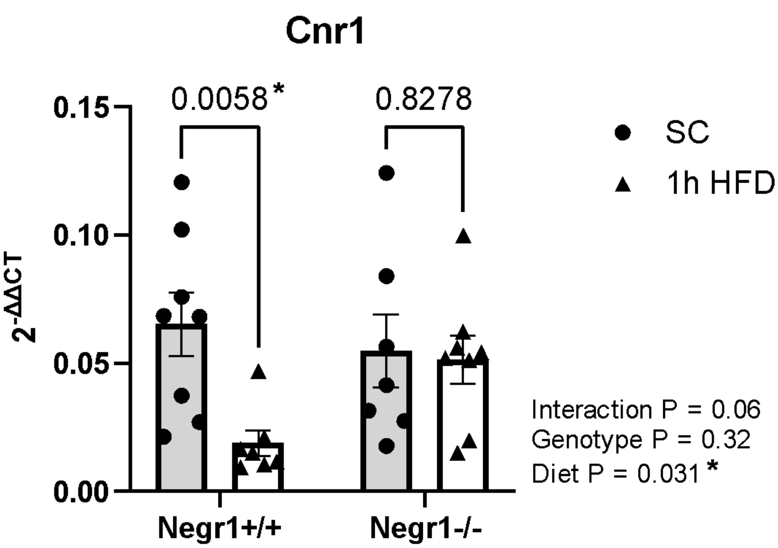

Figure 7. Cannabinoid receptor 1 (*Cnr1*) mRNA expression levels in *wt* and *Negr1*$^{-/-}$ mice in the prefrontal cortex (PFC) with standard chow (SC) and 1- hour exposure to the high-fat diet (HFD).

The relative gene expression of *Mgll* that is involved in the metabolism of the main endocannabinoid in the brain, 2-AG, was neither dependent on the genotype ($F (1, 27) = 0.076$, $p = 0.79$), diet ($F (1, 27) = 1.36$, $p = 0.25$) nor the interaction of diet with genotype ($F (1, 27) = 0.13$, $p = 0.72$) in the PFC-s of *Negr1*$^{-/-}$ and *wt* male mice (Figure 8).

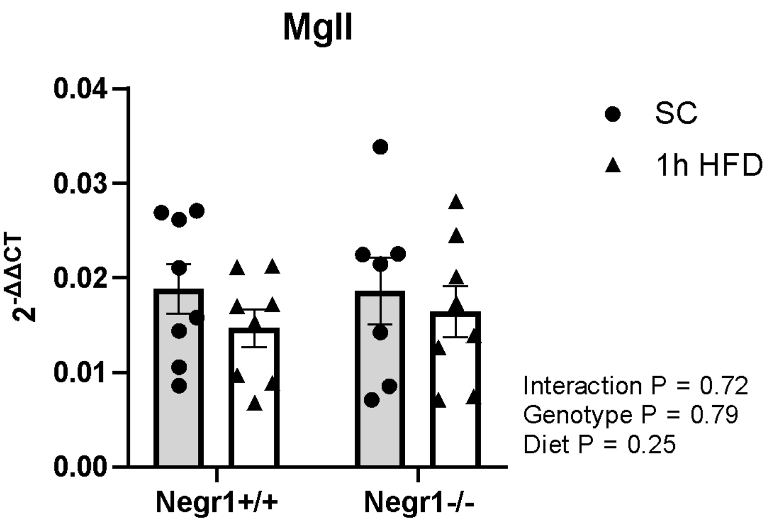

Figure 8. Monoacylglycerol lipase (*Mgll*) mRNA expression levels in *Negr1*$^{-/-}$ and *wt* and *Negr1*$^{-/-}$ mice in the prefrontal cortex (PFC) with standard chow (SC) and 1- hour exposure to the high-fat diet (HFD)**.**

Lastly, measurements of BBB tight-junction protein *Cldn5* mRNA expression levels in the PFC of *Negr1*$^{-/-}$ and *wt* mice PFC revealed a significant effect of diet ($F (1, 27) = 5.32$, $p = 0.029$) and diet interaction with genotype ($F (1, 27) = 6.33$, $p = 0.018$), no significant effect of genotype was observed ($F (1, 27) = 0.34$, $p = 0.56$). *Post- hoc* tests of the 2-way ANOVA confirmed a highly significant ($p = 0.0018$) difference in relative gene expression of *Cldn5* in *wt* mice with different diets and also significant difference ($p = 0.034$) between *Negr1*$^{-/-}$ and *wt* mice on a 1h high-fat diet (Figure 9).

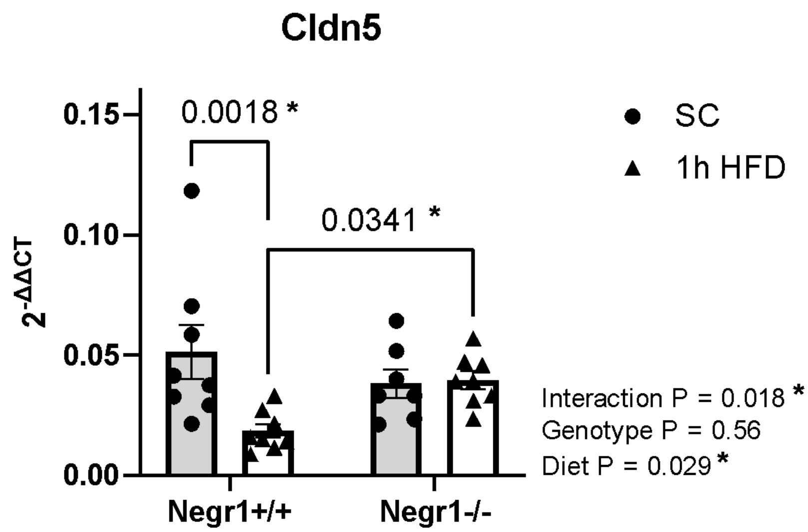

Figure 9. Claudin-5 (*Cldn5*) mRNA expression levels in *Negr1*$^{-/-}$ and *wt* and *Negr1*$^{-/-}$ mice in the prefrontal cortex (PFC) with standard chow (SC) and 1- hour exposure to the high- fat diet (HFD). mRNA expression levels in *Negr1*$^{-/-}$ and wt mice PFC with standard and high fat diet.

6.3. Discussion

Finding differences in transcriptional changes between tissues, disease states or treatment types by detection and characterization of mRNA levels remains one of the most widely used molecular methods in characterizing signaling networks. The acquisition of RNA is the initial and one of the most important steps of ensuring relevant data is reported. Any problems arising from inadequate processing of tissues and cells may result in loss of sample with no do-over (Bustin and Nolan, 2004; Tan and Ylap, 2009).

Chosen and validated extraction method/protocol should give intact and sufficiently pure RNA for further analysis. The work in the current thesis aimed to compare a new column-based RNA extraction kit by Arpengen with the routinely used TRIzol extraction buffer and identify whether one method is superior to another across different types of tissue.

Firstly, the quantity of recovered RNA was found to be very different between the extraction methods, with TRIzol yielding more total RNA in liver and brain tissues. This coincides with previous studies that show that column-based RNA extraction methods have limited ability to extract large amounts of RNA, which could be attributed to limited membrane binding capacity (Tesena *et al.*, 2017; Alves *et al.*, 2016; Sultan *et al.*, 2014). However, the same cannot be said about the RNA extracted from fat tissues, where the membrane-based kit yielded more RNA. Tissues with lower cellular density, such as fat tissue, will have less RNA. So an extraction method that relies a lot on the handling precision will be more difficult to use, as is the case during the extraction of fat tissues with TRIzol where the RNA pellet is not visible, making the collection of washing liquids cumbersome. Although the differences in recovered RNA are significant, sufficient amounts of RNA were gathered from all tissues up to 50 mg weight by the Arpengen kit, while for fat tissues it is recommended that the tissue size be increased if extraction is to be performed with TRIzol.

For the purity of RNA, a spectrophotometer was used to measure absorption values. For the A260/A280 ratios, the difference between the extraction methods is small but the average ratios for all tissues exceeded 1.8, meaning both methods are capable of extracting sufficiently pure RNA for gene expression studies. The absorption ratios for A260/A230 were less ideal and varied a lot across tissues for both RNA extraction methods. Few tissues reached the desired absorption ratio value of around 2, indicating some amount of extraction buffer residuals for both extraction methods. Best results were achieved in brain tissue samples, but for liver and fat tissues further optimization may be required. It should be noted, that there is currently no consensus for a lowest acceptable A260/A230 ratio, as it has been shown in this and previous studies, where even the samples with lowest A260/A230 ratios performed well in RT-qPCR (Qiagen, 2018).

Next parameter that needs to be taken into consideration is the integrity of purified RNA. Little to no degradation is observed in agarose gel photos for all samples extracted with Arpengen kit, while some degradation is present in RNA samples extracted with TRIzol, visible as a low molecular weight band. However, the most important factor in gel electrophoresis integrity control are the ribosomal subunits, which were intact in all extracted tissue samples by both methods. RNA samples exhibiting two ribosomal bands on the gel are assumed to be sufficiently intact, however more precise RNA degradation analysis methods should be used for a more accurate result. RNA sequencing, for example, relies heavily on the integrity of total RNA and the gel might not be as informative, while also requiring a large amount of RNA (Romero *et al.*, 2014). Concerning RNA degradation, other studies have shown that using a more detailed method of micro-capillary electrophoresis to analyze the effect or RNA integrity on PCR efficiency revealed that some degradation is permissible, however the results show higher variability (Fleige and Pfaffl, 2006). According to the gel photos, all samples seem to include genomic DNA contamination, which is the least visible in the fat tissue RNA extracted with Arpengen kit.

RT-qPCR results proved to be difficult to interpret when comparing the two extraction methods. TRIzol extraction seems to work better on the initial examination but there are multiple things to consider before making conclusions. The gel shows the presence of genomic DNA in the extracted RNA but RT-qPCR results in -RT (RNA control) samples do not, which poses a question of presence of components that might inhibit the qPCR reaction in the sample. At the same time, the RT (cDNA) samples show stable amplification by RT-qPCR. This could either stem from mRNA or the genomic DNA contamination could be more accessible for the qPCR reaction. Liver samples extracted with TRIzol show almost no amplification in the RT-qPCR. Compared to RNA extracted from liver tissue by Arpengen kit, there's stable amplification in both RT and -RT samples, making the samples seemingly purer and less degraded, though a similar disruption method was used. In terms of liver tissue in general, the primer choice for the samples was not properly considered, as the chosen genes of interest express at very low levels and primer pairs were improperly designed resulting in unspecific amplification in multiple samples. Adipose tissue sample results in RT-qPCR are in complete contradiction to gel photos. TRIzol extracted samples show a lot more genomic DNA on the gel but amplification in -RT samples is similar to samples extracted with Arpengen.

Results of the RT-qPCRs comparing RNA extraction methods put into question the methods used to evaluate whether acquired RNA is pure enough for downstream experiments. Firstly, NanoDrop gives an estimate of concentration and purity, being unable to differentiate between the type of background contaminants and which nucleic acids are present. Phenol absorption has been shown to overlap with RNA absorption (Unger *et al.*, 2019) resulting in overestimation of concentration, similarly to the presence of genomic DNA. As genomic DNA seemed to be present in almost all samples in this study, one would assume that the concentrations of RNA are overestimated, however it remains unknown to which degree. Therefore, although there are some differences between the Ct values of genes of interest between Arpengen kit and TRIzol extracted samples in different tissue types, it cannot be said whether it is due to quality of the RNA or inaccurate amount of RNA. The assumed inhibition of template amplification in -RT samples which were extracted with TRIzol could be due to the presence of phenol contamination which, as mentioned, results in overestimation of RNA concentration and higher absorption ratios. However, it would then be expected to reflect in RT samples as lower Ct values, which does not seem to be the case in this study.

Several results from the RNA extraction method comparison part of this study do not correspond with the results hypothesized based on previous literature. Many studies show superiority of membrane-based kits compared to TRIzol extraction, due to their ability of former to yield purer RNA free of any contaminants, including genomic DNA, while keeping the RNA well intact (Schwochow *et al*., 2012; Tesena *et al*., 2017; Tavares *et al*., 2011).

With current RNA extraction protocols, DNaseI treatment should be considered if further gene expression analysis involves one exon genes; for multi-exon genes genomic DNA contamination is not an issue in case the primers are designed on exon junctions. In this study DNaseI digestion was tested on samples with various protocols, using on-column digestion and in-solution digestion with concentrations suggested by the manufacturer, and also using different clean-up and inactivation techniques involving heat and EDTA treatments (results not shown). For most samples, RNA extracted with Arpengen kit and then treated with DNaseI followed by cDNA synthesis, RT-qPCR showed almost no amplification of genes of interest, not even housekeeping gene, suggesting inhibition or full degradation of the RNA as a result of the DNaseI treatment. No difference in RT-qPCR results between DNaseI treated and not treated samples was seen in TRIzol samples, which raises concerns about if the DNaseI even worked. Further optimization of the DNaseI treatment that is beyond the scope of this study is needed. But, considering genotype and treatment effects we detect in one-exon genes in the second part of this study, it is possible that even with the presence of some genomic DNA in the sample, the difference in expression of between the groups should still be detectable as we expect genomic DNA contamination to be comparable between samples and not differ between groups.

Overall, the membrane-based Arpengen kit for extracting RNA yields a sufficient amount of intact RNA free of protein contamination with some residuals from the extraction buffer and some genomic DNA. Being that the Arpengen kit uses less hazardous chemicals compared to TRIzol extraction, is less time-consuming, more convenient to use, and produces sufficiently good RNA, the Arpengen kit was used to test its performance in the second part of this study on a larger number of experimental samples from brain tissue.

The second part of this study focuses on an important neuromodulatory system, the endocannabinoid system, and whether the *Negr1* gene might contribute to the development of pathological states in *Negr1*-deficient (*Negr1*$^{-/-}$) mice. Specifically, in this study, the expression of cannabinoid receptor 1 (*Cnr1*) and monoacylglycerol lipase (*Mgll*) with the addition of blood-brain barrier component claudin-5 (*Cldn5*), was compared in the prefrontal cortex (PFC) of wild-type and *Negr1* knockout mice. Additionally, half of these mice were exposed to a high-fat diet for 1h or to standard chow. Short exposure to high-fat food significantly decreases the expression level of CB1 the PFC of *wt* mice. This could be due to a response to changing endocannabinoid levels in the brain, as they are influenced by exposure to high-fat food (Satta et al., 2018). In *Negr1*$^{-/-}$ mice, however, exposure to HFD does not alter the expression of CB1 in PFC. PFC shares a lot of connections to the limbic system and the ECS is abundantly expressed throughout the cortex, so any external changes that have shown to affect the presence of ECS components should be reflected in PFC (McLaughlin et al., 2014). Absence of gene expression changes in the CB1 in *Negr* $^{-/-}$ mice gives reason to suspect dysregulation in the ECS.

This study did not include measurements of endocannabinoid levels in PFC, however the 2-AG metabolizing enzyme MGL showed no changes in expression in PFC of mice in all groups. It would be more informative to include expression levels of endocannabinoid synthesizing enzymes to view whether there are changes in on-demand production of endocannabinoid in the PFC in *wt* and *Negr1*$^{-/-}$ mice with exposure to HFD.

CONCLUSIONS

RNA extraction from tissue samples is a challenging step in gene expression analysis, presenting various problems that may affect result interpretation, so a proper RNA extraction method should be chosen. This thesis focused on the comparison of two RNA extraction methods, one being TRIzol extraction and the second one Arpengen silica membrane extraction kit. To compare the two RNA extraction protocols, several quantitative and qualitative methods were used. Previous methodological studies have identified that column-based extraction kits yield purer RNA, albeit in smaller quantities. In the current thesis, it was found that yield of RNA was higher with TRIzol extraction in brain and liver tissues but not in fat tissues compared to the Arpengen kit. The quality of the RNA extracted with the Arpengen kit was slightly better in terms of contaminants and integrity, however, all samples, regardless of extraction method, seem to have genomic DNA contamination as noted from gel electrophoresis images. Thus, mRNA quality assessment remains inconclusive as there are many variables which prevent proper interpretation. The kit was deemed superior in terms of handling and convenience of use, preventing any loss of RNA and the reproducibility of experiments in different tissues using this method. However, final assessment would require further studies on a larger number of samples with more similar cellular profiles.

The Arpengen kit was used to extract RNA for the gene expression study done in the second part of this thesis on the PFC of wild-type and *Negr1*$^{-/-}$ mice under exposures to different types of food. Changes in transcriptional activity of cannabinoid receptor 1 and monoacylglycerol lipase, with the addition of blood-brain barrier component claudin-5, were compared. It was found that after 1h of high-fat diet exposure, the expression of cannabinoid receptor 1 and claudin-5 was reduced only in wild-type control but not in *Negr1*$^{-/-}$ mice. Monoacylglycerol lipase expression remained unchanged in PFC in all groups of mice. It can be hypothesized based on these results that under certain conditions, in the PFC, *Negr1* affects the normal function of the endocannabinoid system by de-regulation of cannabinoid receptor 1 expression while the expression of the enzyme that breaks down 2-AG remains unchanged. It can be hypothesized that since the profile of claudin-5 expression corresponds to that of cannabinoid receptor 1 results, it is possible that the ECS influences the BBB regulation in the context of acute HFD. Further studies are needed to confirm this hypothesis.

RESÜMEE

RNA eraldamise meetodite võrdlus Arpengeni kiti ja TRIzoli meetodeid kasutades ning Arpengeni kiti kasutamine geeniekspressiooniuuringus

Dana-Karolin Rand

Käesoleva magistritöö eesmärgiks oli võrrelda kahte tüüpi RNA eraldusmeetodeid. Hinnatud olid membraanil põhineval RNA eraldamise kiti ja vedelal baasil põhineval TRIzol-i vahel RNA kvaliteeti ja nende kasutusesefektiivust eri tüüpi kudedele. Samuti püstitati eesmärk välja selgitada kas *Negr1* geenil on seos endokannabinoidi süsteemiga ning vere-aju barjääriga.

Kvaliteetse RNA kasutamine on hädavajalik molekulaardiagnostikas, näiteks geeniekspressiooni katsetes. Kvaliteetse proovimaterjali all peetakse silmas lagunemata RNA-d, mis on saastumata ja piisavas koguses. Madala kvaliteediga RNA-ga töötamine võib kahjustada järgnevate rakenduste eksperimentaalseid tulemusi. Eraldamismeetodi valik võib mõjutada edaspidiste katsete tundlikkust ja spetsiifilisust.

Käesolevas töös võrreldakse mõlemat RNA eraldamise meetodit, Arpengeni kitti membraankolonniga ja TRIzoli lahust, kasutades kvaliteedi ja reprodutseeritavuse hindamiseks spektrofotomeetriat, geelelektroforeesi ja RT-qPCR-i. Eelnevate andmete põhjal püstitati hüpotees, et Arpengeni kitt annab kõrgema kvaliteediga RNA-d. Töö teise osa eesmärk oli kasutada kitti RNA eraldamiseks geeniekspressiooni analüüsiks ajukudedest, et uurida, kas neuronaalse kasvu regulaatori 1 (NEGR1) mõju psüühika häirete etioloogiale võib olla vahendatud muutuste kaudu endokannabinoidsüsteemis (ECS). GWAS uuringutes on täheldatud, et NEGR1 on üks valkudest, millel on väga tugevad geneetilised seosed mitmete psüühikahäirete, eriti meeleoluhäiretega. Kuna nii NEGR1 kui ka endokannabinoidsüsteem mängivad rolli sarnastes psühhiaatrilistes häiretes ja füsioloogilistes protsessides ajus, oli oletatud, et endokannabinoidsüsteemi komponentide ekspressioon NEGR1-puudulikkusega hiirtel erineb.

Käesoleva töö esimeses osas leiti, et RNA saagis oli TRIzoliga ekstraheerimisel aju- ja maksa-, kuid mitte rasvkoes koes suurem võrreldes Arpengeni kitiga. Arpengeni kitiga eraldatud RNA kvaliteet oli saasteainete ja terviklikkuse osas veidi parem, kuid kõik proovid, olenemata eraldamismeetodist, näivad geelelektroforeesi piltide põhjal olevat saastunud genoomse DNA-ga. Kitiga oli siiski võimalik eraldada piisavas koguses RNA-d erinevatest koetüüpidest ning

see oli parema käsitsemise ja kasutusmugavuse osas, mis vältis RNA kadu ja tagas parema reprodutseeritavuse koetüüpide vahel, kuid lõplik hinnang nõuaks täiendavaid uuringuid kus esineb vähem variaablust.

Magistritöö teises osas kasutati Arpengeni kiti, et viia läbi geeniekspressiooni uuring metsiktüüpi ja *Negr1*-puudulikusega hiirte prefrontaalsest koorest erinevatel dieedi tingimustel. Võrreldi kannabinoidiretseptori 1 ja monoatsüülglütserooli lipaasi transkriptsioonilise aktiivsuse muutusi ning lisaks ka vere-aju barjääri komponendi claudiin-5. Leiti, et pärast 1-tunnist kõrge rasvasisaldusega toiduga kokkupuudet vähenes kannabinoidiretseptori 1 ja klaudiin-5 ekspressioon ainult metsiktüüpi hiirtel, kuid mitte *Negr1*$^{-/-}$ hiirtel. Monoatsüülglütserooli lipaasi ekspressioon jäi kõigi rühmade vahel prefrontaal koores muutumatuks. Nende tulemuste põhjal võib oletada, et teatud tingimustel mõjutab NEGR1 prefrontaal koores endokannabinoidsüsteemi normaalset funktsiooni, muutes kannabinoidiretseptori 1 ekspressiooni, samas kui 2-AG-d lagundava ensüümi ekspressioon jääb muutumatuks. Samuti järeldati, et kuna claudiin-5 ekspressiooni taseme muutused sarnanevad kannabinoidiretseptori 1 tulemustele, on võimalik, et endokannabinoidsüsteem mõjutab vere-aju barjääri regulatsiooni läbi claudiin-5 ägeda rasvase toidu kontekstis.

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SUPPLEMENTARY MATERIALS

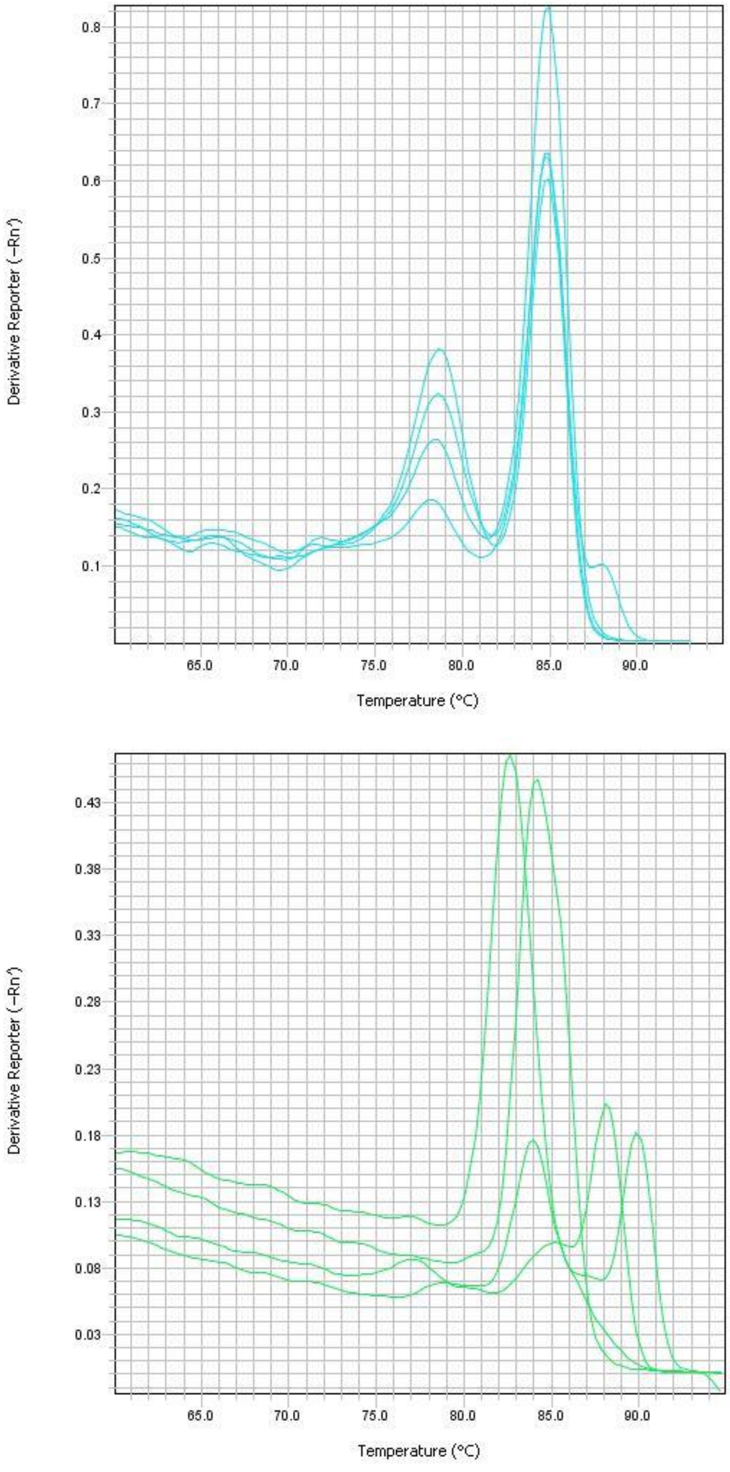

Figure 10. Examples of melt curves that show unspecific amplification in samples during RT-qPCR.

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