Master's thesis

Adenosine modulates L-DOPA level during physical activity though NR4A2 (Nurr-1) – TYROSINE HYDROXYLASE–L-DOPA pathway

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Content:

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Abstract:

Previously our research team found nine genes (ATF3, NR4A2, NFIL3, NR4A3, MAFF, SIK, SLC2A3, MYC, and SOCS3) that are differentially expressed in sedentary and physically active people. Another study of our team showed an affinity level of gut derived metabolites that interacted with these nine proteins. One of the strongest binding activities was observed between adenosine and NR4A2. NR4A2 is a nuclear orphan factor responsible for adaptation to physical activity and neuronal signaling. As a step towards understanding the mechanism of gut derived metabolites affecting mental function such as motivation to physical activity through gene modulation, this study was performed. To investigate the regulation of L-DOPA level through the NR4A2-TH-L-DOPA pathway through Adenosine, the qPCR analysis and Western blot analysis were used. Rhabdomyosarcoma (RD CCL-136) cells were used as a platform for gene expression analysis. The results showed the ability of adenosine to initiate expression of NR4A2. Further investigation of the proposed mechanism should be performed to clarify expression of NR4A2 in a time and dose dependent manner. The study will elucidate a broader understanding of gut-derived adenosine's effect on NR4A2-TH-L-DOPA pathway and the potential mechanism of motivation it regulates.

Introduction:

Physical activity modulates gene expression through DNA methylation (Geiger et al., 2024), and our previous study showed nine genes differentially expressed in sedentary individuals and elite athletes. These genes are responsible for a variety of functions such as regulation of inflammation, metabolism, adaptation to stress and immune response. The genes and its known functions are listed in Table 1.

Table 1. Differentially expressed genes in sedentary and physically active individuals and its functions.

No	Gene	Gene Symbol	Function	Reference
1	Activating Transcription Factor 3	ATF3	Maintains the quiescence of skeletal muscle stem cells	Zhang et al., 2023
2	Nuclear Receptor Subfamily 4, Group A	NR4A2 and NR4A3	Transcription factors involved in metabolic regulation (glucose), inflammation, and cellular stress responses	Prince et al., 2017
3	Nuclear Factor Interleukin 3 Regulated	NFIL3	Binds to ATF sites, regulates genes involved in immune responses such as NK cells and T cells	Kim et al., 2019
4	MAF BZIP Transcription Factor F	MAFF	Oxidative stress response, detoxification, and metabolism	Wang et al., 2020
5	Salt-Inducible Kinase	SIK	Energy metabolism, immune response, neurophysiology, implicated in muscle insulin sensitivity	Jagannath et al., 2023
6	Solute Carrier Family 2 Member 3	SLC2A3 (GLUT3)	Glucose transport	NLM, 2024
7	MYC Proto-Oncogene	MYC	Regulates genes involved in metabolism and protein synthesis for muscle growth and adaptation	Jones et al., 2024
8	Suppressor of Cytokine Signaling 3	SOCS3	Controls inflammatory responses during muscle injury and regeneration	Luo et al., 2024

These genes, ATF3, NR4A2, NFIL3, NR4A3, MAFF, SIK, SLC2A3, MYC, and SOCS3 have been investigated for interconnection using STRING (Szkarczyk et al., 2019) (Figure1), a bioinformatics tool used for gene interaction analysis. All the listed proteins are assumed to play a role in physical activity, but the only protein responsible for physical activity and neuronal regulation is NR4A2. It regulates energy metabolism, mitochondrial function (Pearen & Muscat, 2010) and responsive to

neuronal activity (Volakakis et al., 2015). As a potential regulator of the adaptation to physical activity through activation of the neuronal pathway this protein is investigated in this research.

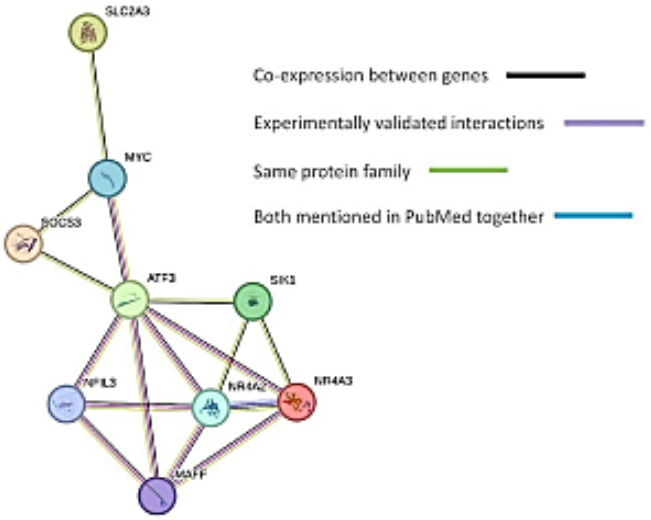


Figure 1. STRING analysis of the potential interactions between nine genes.

Gut microbiome is represented by a variety of microorganisms producing different metabolites such as bile acids, short chain fatty acids, phenols, etc. (Wu et al., 2021) At the same time, the composition of microbiome regulates the metabolism of living organisms, which was shown by fecal transplantation in mice to demonstrate the metabolic changes (Liu et al., 2020). Additionally, the gut-microbiome derived metabolites regulate exercise motivation regulation of dopamine levels through activation of TVRP1 and CD1 receptors and increase levels of monoamine oxidase (MAO) that inhibits dopamine degradation (Dohnalova et al., 2022). To sum up, the gut derived metabolites have a complex and comprehensive range of actions through the microbiome-gut-brain axis affecting mood (Guo et al., 2022), motivation (Guo et al., 2022), and athletic performance (Lensu & Pekkala, 2021).

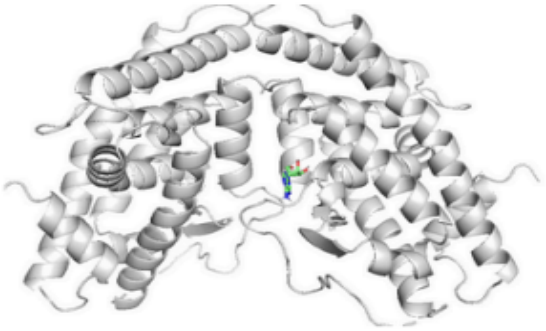


Figure 2. Adenosine binding to NR4A2 3D model

According to molecular docking simulation, shown by our group previously, there are 32 microbiome-derived metabolites that can bind the above-mentioned nine proteins at different affinities. Notably, one of the highest affinities was shown between adenosine and NR4A2 protein, -6.636 -5.858 kcal/mol (Figure 2). The full list of the gut derived metabolites and the range of affinity level is presented in Table 2.

Table 2. Results of molecular docking analysis of NR4A2 protein and gut-derived metabolites binding ability level.

№	Metabolite	Range min to max(kcal/mol)		Top 5 Amino Acids (Frequency)
1	Deoxycholic acid	-8.047	-6.811	ILE 498 (5), TYR 453 (4), ASN 497 (4), GLN 494 (3), ARG 515 (3)
2	ursodiol	-7.889	-6.642	GLN 528 (7), GLN 494 (7), LEU 559 (3), ARG 515 (3), GLU 445 (3)
3	adenosine	-6.636	-5.858	GLU 445 (6), TYR 453 (6), ARG 515 (5), HIS 372 (4), ILE 498 (4)
4	(PAG)Phenylacetylglutamine	-6.532	-5.683	ARG 370 (7), GLN 494 (5), ILE 498 (4), TYR 453 (3), ILE 500 (2)
5	Indole -3 propionic acid	-6.252	-5.64	ARG 370 (6), TYR 453 (4), ILE 498 (4), ARG 454 (3), PRO 377 (2)
6	Indole -3 lactic acid	-5.976	-5.499	ARG 370 (6), GLU 445 (6), ARG 515 (6), ARG 454 (4), GLN 494 (4)

7	N-Oleoyldopamine	-5.968	-5.279	ALA 510 (4), ARG 515 (3), GLU 445 (3), ASN 497 (3), THR 513 (3)
8	indoxyl sulfate	-5.928	-5.31	ARG 515 (3), THR 513 (3), VAL 362 (2), SER 363 (2), LEU 559 (2)
9	NADA	-5.921	-5.312	ARG 370 (3) GLU 445 (3) TYR 453 (2) ASN 497 (3), ILE 498 (3)
10	N-Methylnicotinamide	-5.678	-5.017	PHE 592 (5), LEU 444 (3), GLU 440 (2), LEU 591 (2), THR 595 (2)
11	D-Xylose	-5.542	-4.503	ASP 499 (5), VAL 539 (4), ARG 515 (3), GLU 445 (3), LEU 559 (2)
12	D-Glucose 6-phosphate	-5.484	-4.878	GLU 445 (6), THR 513 (5), ASP 374 (5), ALA 510 (4), ARG 370 (4)
13	Dihomo-gamma-linolenic acid	-5.293	-4.402	GLN 494 (5), TYR 453 (4), ASN 497 (3), ILE 498 (3), ARG 370 (2)
14	Isoleucine	-5.243	-4.083	ARG 515 (4), LEU 591 (3), THR 513 (3), LEU 444 (3), THR 595 (3)
15	2 methyl 2 pyridone 5 carboxamide (2PY)	-4.971	-4.518	GLU 445 (5), ARG 515 (4), SER 366 (3), PHE 489 (3), SER 363 (3)
16	Eicosadienoic acid	-4.824	-4.462	ASN 497 (3), ARG 515 (3), GLN 494 (2), ILE 498 (2), THR 567 (2)
17	OEA	-4.813	-4.268	ARG 370 (6), GLN 494 (5), ASN 497 (4), GLU 445 (3), THR 513 (3)
18	3-phosphoglycerate	-4.776	-4.176	ARG 515 (6), ASP 499 (5), GLU 481 (4), GLN 568 (4), SER 363 (3)
19	PEA	-4.705	-4.267	ARG 515 (4), GLN 494 (3), SER 441 (2), GLU 445 (2), ASN 533 (2)
20	Acetoacetate	-4.666	-3.841	ARG 370 (4), LEU 591 (4), PHE 592 (4), SER 366 (3), SER 501 (3)
21	Valine	-4.658	-4.157	LEU 444 (5), LEU 591 (4), PHE 592 (4), GLU 440 (2), THR 595 (2)
22	Acetyl-L-Carnitine	-4.631	7.111	HIS 372 (3), PRO 377 (3), ARG 454 (3), MET 379 (3), ASP 374 (2)

23	leucine	-4.527	-4.044	ARG 515 (3), HIS 372 (2), MET 379 (2), TYR 453 (2), HIS 538 (2)
24	phosphoric acid	-4.408	-3.49	SER 501 (4), ASP 499 (4), ILE 500 (3), GLU 445 (3), ARG 572 (3)
25	carnitine	-4.321	-3.914	ASP 499 (4), VAL 539 (4), ARG 370 (3), SER 441 (3), ARG 454 (2)
26	threonine	-4.198	-3.771	ASP 499 (4), THR 513 (3), LEU 591 (3), TYR 551 (3), GLU 445 (3)
27	Butyrate	-4.058	-3.635	SER 501 (3), LEU 591 (3), THR 595 (3), SER 363 (3), ARG 370 (2)
28	2,3-butanediol	-4.051	-3.813	LEU 591 (4), PHE 592 (4), SER 501 (3), ASN 542 (3), ILE 588 (2)
29	glycine	-3.839	-3.091	SER 366 (4), SER 501 (4), HIS 538 (4), ILE 500 (3), ASP 499 (3)
30	acetate	-3.471	-2.699	PHE 592 (4), ARG 370 (4), ASP 499 (3), LEU 552 (2), SER 501 (2)
31	TMAO	-2.91	-2.528	ARG 515 (3), TYR 551 (3), GLU 445 (2), GLY 480 (1), GLU 481 (1)

Our particular attention was focused on the gut derived adenosine, since adenosine is a nucleoside that plays a crucial role in energy metabolism, blood flow and fatigue. During prolonged physical activity, the breakdown of ATF in skeletal muscle causes an increase of adenosine level in muscle and brain (Dworak et al.,2007). The opinion that adenosine causes fatigue and feeling of low energy is supported by Davis et al, 2003, however, the modern research of Zhu H., 2024 revealed a reversible effect. The experimental animal study showed anti-fatigue effect and improvement of exercise performance in mice through AMPK/PGC-1a signaling pathway, which enhance mitochondrial function and energy metabolism. The controversial and context dependent effect of adenosine, its high binding activity to NR4A2, a gene that contributes

to early response to stress and physical activity as shown by our team previously, contributed to the idea investigating adenosine beyond the first two bile acid metabolites (ursodiol and deoxycholic acid) which showed higher affinity.

Adenosine derived by gut microbiomes has a very diverse range of action: it is associated with idiopathic short stature in children (Yan et al., 2024), intestinal protection (Stepanova & Aherne, 2024) and metabolism control through purinergic signaling (Li et al., 2023).

One more important role of adenosine and its analogue is to activate Nuclear orphan receptors (NR4A1, NR4A2 and NR4A3) through activation of adenosine receptors (Zhang et al., 2010). Meanwhile, the NR4A2 has been shown as an activator of tyrosine (TH) (Kim et al., 2003), a gene encoding the enzyme that converts tyrosine to L-DOPA which is then converted to dopamine (Molinoff & Axelrod, 1971) by aromatic L-amino acid decarboxylase (AADC) enzyme (Mura et al., 1995). The presented mechanism assumes regulation of motivation level to physical activity through adenosine released by gut microbiome (Figure 3).

Adenosine outside the cell acts as a signaling molecule activating G-protein-coupled adenosine receptors and regulates the intracellular cascade cAMP-PKA- CREB (Quarta et al., 2004). Activation of this cascade lead to phosphorylation of ERK, a molecule that induces intranuclear transcriptional program of NR4A family (NR4A1/Nur77, NR4A2/Nurr1, and NR4A3/Nor1) and acting as transcriptional factor (Hawk & Abel, 2011). Synthetic agonist of adenosine receptors shows an increase of NR4A2 expression level, showing the specific pathway of NR4A2 activation through adenosine receptors (Murphy et al., 2010). This link and mechanism indicate the role of adenosine outside the cell as a regulatory agent particularly in neuronal cells (Hawk et al., 2011).

In neuronal cells, the NR4A2 plays an important role in functional maturity of dopaminergic neurons, since it regulates the level of tyrosine hydroxylase enzymes in a rate limiting manner (Decressac et al., 2013). The NR4A2 could potentially bind to 13 sites of Tyrosine hydroxylases' promoter and upstream signaling (Eells et al., 2012). The experimental study, inducing NR4A2 genes, showed an increase of TH mRNA, supporting the idea that NR4A2 initiates a cascade of TH expression. Therefore, adenosine-NR4A2-TH pathway represents an important role in neuromodulatory signaling and regulates dopamine synthesis (Decressac et al., 2013).

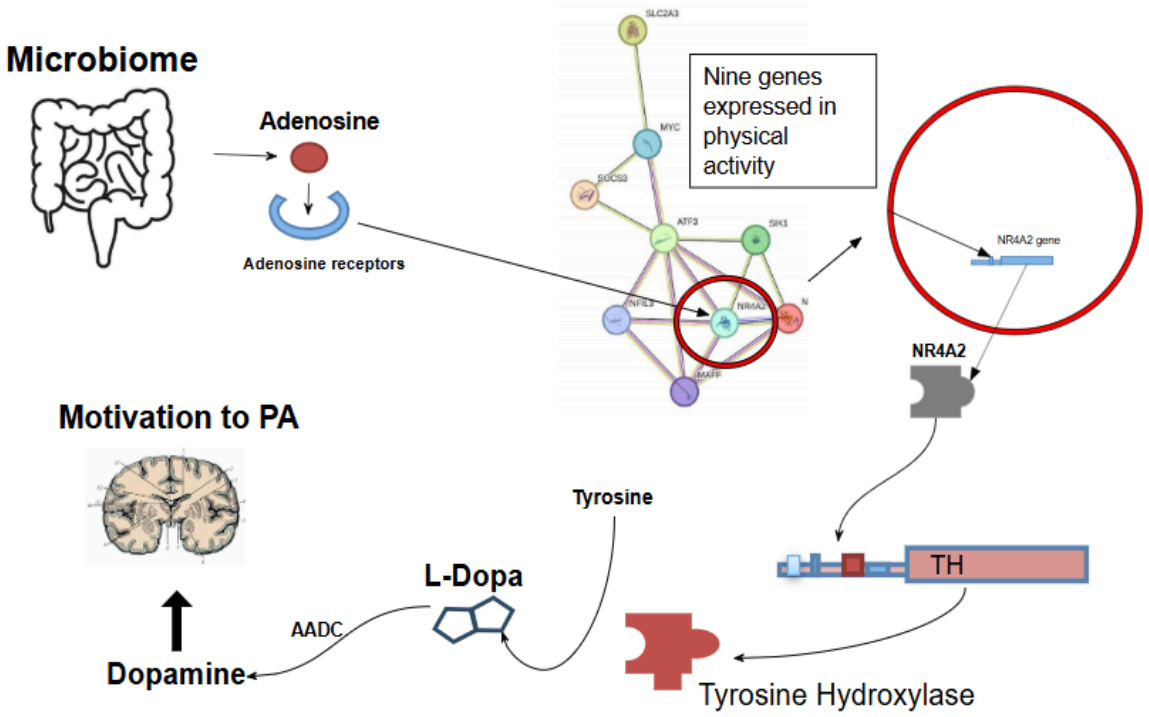


Figure 3. Mechanism of possible regulation of motivation through NR4A2–TH–L-DOPA pathway by gut-derived adenosine.

The dependency of NR4 family gene expression and Dopamine levels were shown via negative feedback regulation in mice through drugs affecting D2- receptors (Eells et al., 2012). Our current

study is aimed to investigate a mechanism of motivation to physical activity regulation through the NR4A2-TH-L-DOPA pathway activated by gut derived adenosine.

Premise:

Gut derived metabolites could regulate metabolism through modulation of the above nine genes. Further in vitro experiments are needed to show if microbiome derived metabolites, i.e. adenosine, regulate motivation to physical activity through the NR4A2-TH-L-DOPA pathway.

Objectives/Research Question/Hypothesis:

Objectives- *To study effect of Adenosine on NR4A2-Tyrosine Hydroxylase-L-DOPA pathway*

Research Question- *Does adenosine affect NR4A2-Tyrosine Hydroxylase-L-DOPA pathway?*

Hypothesis- *Adenosine affects NR4A2-Tyrosine Hydroxylase-L-DOPA pathway*

Specific aims:

1. To receive consent from NU Biological and Chemical Safety Committee and IREC

2. To test effect of Adenosine on the NR4A2- Tyrosine Hydroxylase- L-DOPA pathway by growing the Rhabdomyosarcoma (RD CCL-136) in the presence and absence of adenosine and measuring the levels of:

a) NR4A2 and Tyrosine Hydroxylase by qPCR and Western blot

b) L-DOPA by spectrophotometry

3. To perform statistical analysis using Student's t-test to measure significance of results between adenosine treated and not treated cells.

Experimental design:

The in vitro experiments were performed on Rhabdomyosarcoma cells (RD-CCL) with and without Adenosine treatment. The gene expression of NR4A2, protein NR4A2 synthesis and L-DOPA levels was analyzed using qPCR, Western blot and Spectrometry analysis, respectively.

The ethical approval from Nazarbayev University Biological and Chemical Safety Committee and Nazarbayev University Institutional Research Ethics Committee was not needed since the experiment was based on commercially available human cells (RD CCL 136, ATCC).

Materials:

Reagents:	**Manufacturer:**	**Catalog number:**
DMEM - high glucose	Sigma-Aldrich	D5796
Fetal Bovine Serum	Sigma-Aldrich	F2442-500ML
Penicillin-Streptomycin	Sigma-Aldrich	P4333
Trypsin-EDTA solution	Sigma-Aldrich	T4049-100ML
Phosphate-buffered saline	ThermoFisher	003002
Tamoxifen	ThermoFisher	J63509.03
Dimethyl sulfoxide	Sigma-Aldrich	276855
Tris	Sigma-Aldrich	10708976001
Sodium chloride	Sigma-Aldrich	S9888
Triton X-100	QazMura	QM5264- 500ML
Sodium deoxycholate	Sigma-Aldrich	BCCB0916
Sodium dodecyl sulfate	QazMura	QM4700-500G
Ethylenediaminetetraacetic acid	AppliChem	A1103,1000

Halt™ Protease and Phosphatase Inhibitor Cocktail, EDTA-free (100X)	ThermoFisher	78445
TGX FastCast Acrylamide Kit, 10%	Bio-Rad	1610173
Ammonium persulfate	Sigma-Aldrich	A3678-25G
TEMED	Bio-Rad	1610801
2x Laemmli sample buffer	Bio-Rad	1610737
2-Mercaptoethanol	Sigma-Aldrich	M6250
PVDF Transfer Membrane 0.45um	ThermoFisher	88518
Precision Plus Protein Dual Color Standards	Bio-Rad	1610374
NR4A2 Monoclonal Antibody	ThermoFisher	5A9B9
Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP	ThermoFisher	31430
EZ-ECL Detection Kit	Sartorius	20-500-120
PowerUp™ SYBR™ Green Master Mix	Thermo Fisher Scientific	A25742
High-Capacity cDNA Reverse Transcription Kit	Thermo Fisher Scientific	4368814
PureLink RNA Mini Kit	Monarch	12183018A
Nuclease-free Water	Thermo Fisher Scientific	AM9937

Cell culture:

Rhabdomyosarcoma cells (RD-CCL 136) were cultured in Dulbecco's Modified Eagle Medium (DMEM), which contained of 4500mg/L glucose, 10% fetal bovine serum (FBS), and 1% penicillin- streptomycin. Cells were incubated at 37^{o}C with 5% CO_2 for 2-3 days to reach 80% confluence, and reseeded for 6-, 12-well plates using 0.05% trypsin- EDTA solution for detachment.

Drug treatment:

When cell confluence reached 50-60%, the treatment with Adenosine was performed.

10mM stock solution of Adenosine prepared using 2.67 g of Adenosine powder (Mayer) and 1mL of distilled water. Working stock for treatment was prepared by dissolving powder into distilled water exactly before treatment due to the fast degradation rate of Adenosine. The cells were treated with 100µM, 200uM, 300uM, 400uM and 500µM Adenosine concentration for 3 and 12 hours. Control wells included: a) cells treated with media and same amount of distilled water used for Adenosine dissolution (as a vehicle control); b) cells treated with media (untreated control). Each condition is performed in two biological replicates for each experiment to ensure reproducibility, consistency and robustness of the results.

qPCR:

Cells from 6-,12- well plate after 3- and 12-hours treatment collected into a 1.5 ml Eppendorf tube using 0.05% trypsin-EDTA solution for detachment and further neutralization with media. Then, centrifuged at 500xg at room temperature for 5 minutes and threw away the supernatant. The cell platelet washed with PBS and then again centrifuged at 500xg at the room temperature.

For RNA extraction, RNA MiniKit is used following the manufacturer instruction (Thermo Fisher Scientific). Cells were lysed using a lysis buffer containing 2-mercaptoethanol and followed by 96% ethanol addition for RNA binding for the silica column. After washing, RNA was eluted in RNAse-free water and checked via Nanodrop 2000c Spectrophotometer for concentration and purity. Samples were stored on ice and at -80C until the next stage.

For cDNA synthesis, 20 uL total solution of SuperScript Reverse Transcriptase, 5ug of total RNA, oligo(dT) primers to NR4A2, dNTPs used in accordance with manufacturer protocol. The reaction was performed using standard protocol of our laboratory (Appendix).

qPCR was performed using QuantStudio 6 Flex using Maxima SYBR Green and ROX qPCR Master Mix. 10µL of solution contains 5µL of SYBR mix, 0.5µL of NR4A2 reverse and forward primers, 3uL nuclease free water and 1µL cDNA template (440ng/uL). After 40 cycles the CT parameters are received.

Western blot:

Cells from a 6-well plate after 12 hours of treatment collected for protein extraction and washed with ice-cold PBS. The RIPA lysis buffer is used with a protease inhibitor cocktail in accordance with manufacturer instructions to prevent protein degradation. Further, cells were incubated for 20 minutes with gentle agitation. The solution gained after collecting cells by scraping, centrifuged in small tubes at 16.000xg for 15 minutes at 4^{o}C. Supernatant with protein was transferred to a new tube with 2x Laemmli sample buffer containing 5% 2-mercaptoethanol and boiled at 95^{o}C for 5 minutes.

Same amount of protein resolved on 10% SDS-PAGE gel prepared using TGX FastCast Acrylamide Kit (Bio-Rad) and electrophoresed at 110V for one hour. Proteins were transferred onto

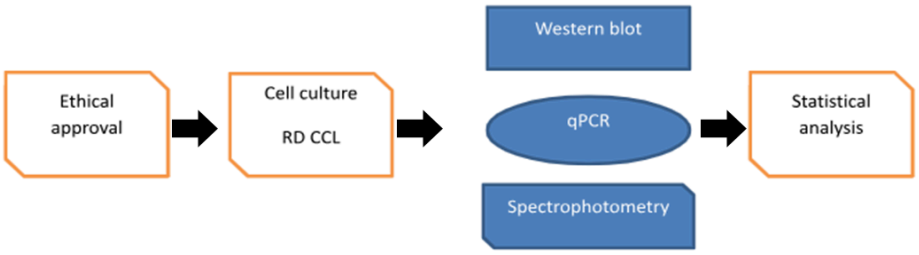
the PVDF membrane (ThermoFisher) using a semi-dry method for 30 minutes at 25V. The membrane is blocked with Everyblot blotting solution for 1 hour at room temperature for unspecific binding prevention. Overnight incubation at +4C of membrane with primary antibodies to NR4A2 was followed with washing with PBS-T 4 times on the next day. Incubation with HRP-conjugated secondary antibodies last for 1 hour at room temperature. The ECL chemiluminescence substrate Sartorius was used for detection and Bio-Rad ChemiDoc for visualization of the protein bands.

Statistical analysis:

For data standardization and visual presentation, the ddCT and Fold Change methods were used (Livak, K. J., & Schmittgen, T. D.,2001).

Student's t-test was used to determine statistical significance between gene expression in treated and untreated cells.

A *p* value less than 0.05 is used to evaluate the statistical significance of results.



Results

Treatment with Adenosine showed a time- and dose-dependent effect on the expression of NR4A2 in Rhabdomyosarcoma (RD CCL-136) cells with statistical significance (p-value<0.05). The

fold Change of NR4A2 expression in cell samples treated with Adenosine showed gradual increase with concentration after 3 hours of treatment.

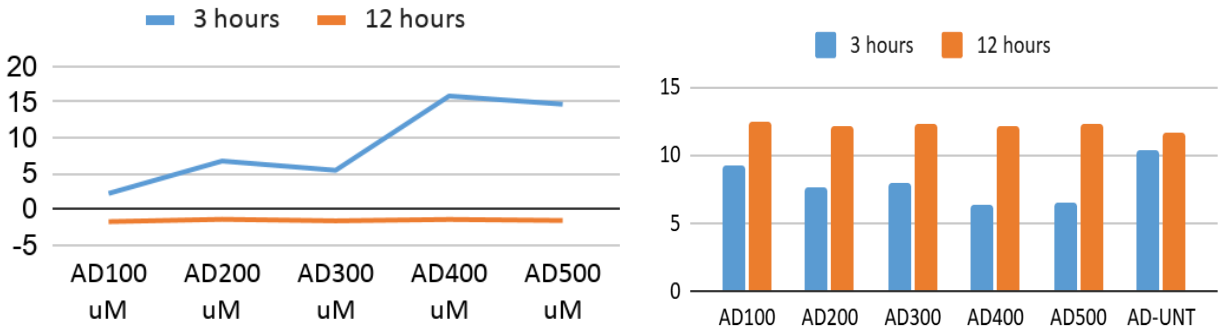


Figure 4. Fold Change and ddCT of NR4A2 expression after treatment with Adenosine 100uM, 200uM, 300uM, 400uM, 500uM concentration during 3 and 12 hours.

The peak expression was observed within 400uM-500uM Adenosine concentrations, which are 15.82 and 14.67 respectively. That is three times higher than it shows in 100uM, 200uM and 300uM Adenosine concentrations, which are 2.19, 6.72 and 5.43 respectively. The 12 hours of treatment with Adenosine shows that NR4A2 expression decreased in comparison with untreated control. The average value of Fold Change is within 0.2-0.3 with no dependency in concentration.

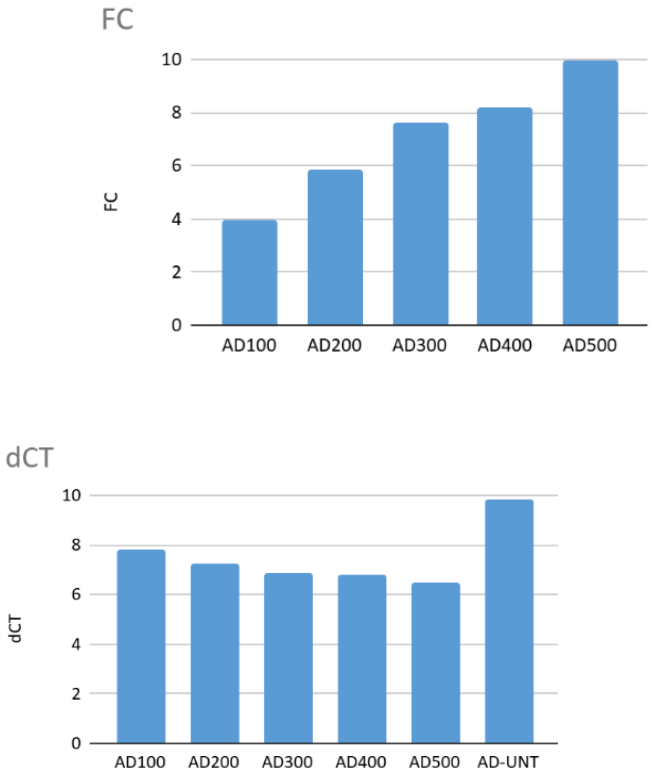


Figure 6. Fold change and ddCT of NR4A2 expression after treatment with Adenosine 100uM, 200uM, 300uM, 400uM, 500uM concentration during 3 hours

The consecutive independent experiments with adenosine treatment for 3 hours showed the same dose dependent pattern indicating the reproducibility of the experiment (Figure 6). Assuming the whole experimental data, adenosine shows statistically significant effect in all concentrations, with Fold Change from 2.9 to 12.17 and p-value < 0.05 (Figure 5).

Sample	Mean ΔCt (NR4A2 - RPL13A)	SD ΔCt	Fold Change (vs untreated)	p-value (vs untreated)
Untreated	10.096	0.4	1	
AD100	8.536	0.84	2.96	0.029
AD200	7.446	0.22	6.32	0.002
AD300	7.41	0.57	6.48	0.009
AD400	6.587	0.45	11.45	0.001
AD500	6.501	0.14	12.17	0

Figure 5 The RNA expression level of NR4A2 quantified using qPCR analysis. RPL13A is used as a housekeeping gene. Statistical significance evaluated using Student's T-test, p-value <0.05.

The Western blot analysis of NR4A2 protein was performed on cell samples treated with Adenosine 100 uM and 300 uM concentration.

Figure 7. Protein bands of Adenosine treated RD cells with two biological replicates.

The housekeeping protein which is alpha tubulin showed a band on 50 kDa level and showed clear and consistent bands. Samples treated with Adenosine had shown blur and inconsistent bands in both concentrations and untreated control cells (Figure 5).

Discussion:

In this study, the effect of Adenosine on expression of NR4A2 in Rhabdomyosarcoma (RD CCL-136) was studied using qPCR and Western blot analysis. The results showed a dose and time dependent effect of NR4A2 expression in qPCR analysis. The same pattern of NR4A2 expression was previously shown in human mast cells, where the peak of NR4A2 expression was observed

within the first 6 hours (Zhang L., et al., 2010). Zhang L. also showed the mechanism of NR4A2 expression through the adenosine receptors ratio combination. The article presented evidence that A2A receptors decrease the expression of NR4A2, whereas A2B and A3 significantly amplify the NR4A2 expression. The mechanism involves the PEK-MEC-ERK1/2 and positively affects expression of NR4A2 and NR4A3. Another study showing Adenosine effect on the expression of NR4A2 was reported by Crean D. et al, 2015, in monocytic cells. The mechanism of NR4A2 expression activated by adenosine receptor agonist (NECA) showed a different pattern in context of adenosine receptor functional specificity. The stimulation of adenosine receptors A2A with agonist drugs affected the NR4A2 expression in an upregulatory way that is contradictory with the mechanism in human mast cells (Zhang L., 2010). In our study, we have not checked for specific functional effects of different adenosine receptors, however, using the human protein atlas database, we found that adenosine receptors in Rhabdomyosarcoma (RD CCL-136) cells are presented mostly by A1-receptors in a ratio 71:3.1:13.8:0, A1, A2A, A2B and A3 respectively. Thereby, the effect of Adenosine to expression of NR4A2 in our study could be throughNR4A2 expression affected via A1 adenosine receptors. One potential future direction could be a consecutive antagonism of adenosine receptors followed by evaluation of NR4A2 expression.

A noticeable finding is that expression of NR4A2 after 12 hours of treatment is decreased with no correlation to concentration. One possible explanation shown by Crean D. is binding activity of NR4A2 with NF-kB/p65. Since the A2A receptors activation with Adenosine initiates TLR4/LPS- NF-κB/p65 cascade, the abundance of NR4A2 proteins inhibit phosphorylation of NF-kB and decreases expression of NR4A2 in monocytic cells (Crean D. et al, 2015). Another negative feedback mechanism was described by Misund K. in 2013. The mechanism was observed

in Adenocarcinoma cells where the expression of NR4A2 was induced by Gastrin. The consecutive evaluation of NR4A2 after eight hours of continuous Gastrin treatment showed 50-70% decrease of NR4A2. This was explained by ICER and Zfp36l1 protein expression induced by abundance of NR4A2. The valuable detail is that adenocarcinoma cells express A2B and A3 adenosine receptors (Misund K. et al, 2013) that correlates with the pattern of Zhang L's study. Nevertheless, Zhang's and Misund's experiments were performed with different drugs and on different cell lines the expression of NR4A2 showed a similar pattern. The common mechanical chain in two previous studies drives the discussion of feedback mechanisms in the Rhabdomyosarcoma cells mostly through cAMP rather than ICER and Zfp36I1, but should not be limited to that. This idea is supported by the similar pattern of adenosine receptors in Rhabdomyosarcoma, Human Mast Cells and Adenocarcinoma. This conclusion is consistent but strongly contradicts with the fact that adenosine A1 receptors inhibit cAMP (Crean et al, 2015). That again refers to the necessity for future experiments with detailed and accurate investigation of A1 adenosine receptors' effect on expression of NR4A2 using consecutive antagonism of other receptors.

Expression of TH could be potentially detected within a 12-24 hours interval after treatment. Zhang L, showed that NBRE binds to NR4A2 after 12 hours and decreases after 24 hours after treatment with NECA (adenosine analogue). The interesting results gained within our experiments also require further validation. The experiment was performed twice with the same condition. The only level the band appeared after incubation with NR4A2 primary antibody was 37 kDa. The technical recommendations of antibodies to the NR4A2 manufacturer proposed detection of protein bands on 66- 70 kDa level. The expression of bands at 37 kDa is possible in transfected lysate in accordance with the Abnova manufacturer's instruction. Previously, the

degradation of NR4A2 protein was shown within 3 hours through ubiquitin- proteasome system (Alvarez-Castelao, B et al, 2006). Thereby the observed bands could represent sub-particles of NR4A2 protein with antigen determinants. The absence of a band at 70 kDa is unclear and requires further optimization of reagents and conditions. Since there were no previous experiments on Rhabdomyosarcoma (RD CCL-136) cells, no fulltime calibration for NR4A2 expression by hours, it is reasonable to assume that it could be a result of a protein degradation process, initiated by a negative feedback mechanism. That explains low molecular weight and refers to the unknown mechanism of NR4A2 expression mechanism through A1 adenosine receptors and negative feedback mechanism. The further optimization of Western blot steps and reagents (antibodies) could strengthen or completely deny this assumption.

Limitations and future directions

The research was performed within seven months from proposal writing to oral presentation of the results. The study began with and included optimization of experimental conditions, reagents order and mastering laboratory skills. The time constraints were anticipated and significantly impacted the completion of the master's thesis experiments.

Future directions could include all the planned experiments within the proposed hypothesis which are TH evaluation using qPCR and Western blot; and L-DOPA detection using spectrometry. Following this, further investigation of NR4A2 expression mechanism through A1-adenosine receptors should be investigated as well as a negative feedback mechanism of NR4A2 protein level control.

Conclusion

The results show that Adenosine could potentially modulate L-DOPA level trough NR4A2-TH- L-DOPA pathway. The hypothesis requires further experimental investigation and validation. Feedback regulation of NR4A2 expression and A1- adenosine receptors mediated NR4A2 expression requires further investigation.

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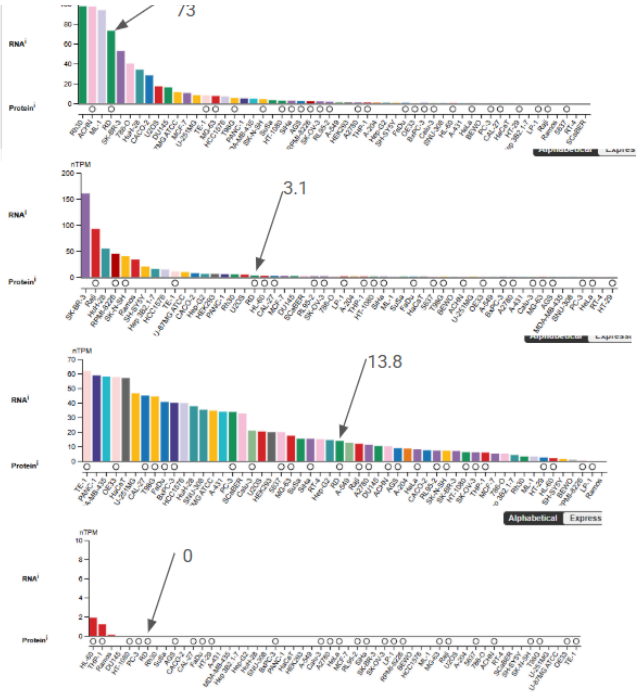
Appendix

Table 4. Expression of Adenosine receptors in Rhabdomyosarcoma cell line (RD CCL-136) by The Human protein atlas.

THE HUMAN PROTEIN ATLAS

Expression of Adenosine receptors in RD cells:
A1- ADORA1
A2A-ADORA2A
A2B-ADORA2B
A3- ADORA3



Variable	A1-R	A2a-R	A2b-R	A3-R
Skeletal muscle	+	+	++	++
RD cell line	+++	++	++	+
	73	3.1	13.8	0

Table 5. Protocols for cDNA and qPCR

Samples/primers	SOCS3	NFIL3	RPL13A
HEK cDNA	1ul - cDNA 4ul - water 4ul - SYBR green 0.5ul - FP 0.5ul - RP	1ul - cDNA 4ul - water 4ul - SYBR green 0.5ul - FP 0.5ul - RP	1ul - cDNA 4ul - water 4ul - SYBR green 0.5ul - FP 0.5ul - RP
NTC	5ul - water 4ul - SYBR green 0.5ul - FP 0.5ul - RP	5ul - water 4ul - SYBR green 0.5ul - FP 0.5ul - RP	5ul - water 4ul - SYBR green 0.5ul - FP 0.5ul - RP

PROTOCOLS

I. First Strand cDNA Synthesis

After thawing, mix and briefly centrifuge the components of the kit. Store on ice.

1. Add the following reagents into a sterile, nuclease-free tube on ice in the indicated order:

Template RNA	total RNA *or* poly(A) mRNA *or* specific RNA	0.1 ng - 5 µg 10 pg - 0.5 µg 0.01 pg - 0.5 µg
Primer	Oligo $(dT)_{15}$ primer *or* Random Hexamer primer *or* gene-specific primer	1 µL 1 µL 15-20 pmol
Water, nuclease-free		to 12 µL
	Total volume	12 µL

2. Optional. If the RNA template is GC-rich or contains secondary structures, mix gently, centrifuge briefly and incubate at 65°C for 5 min. Chill on ice, spin down and place the vial back on ice.
3. Add the following components in the indicated order:

5X Reaction Buffer	4 µL
RiboLock RNase Inhibitor (20 U/µL)	1 µL
10 mM dNTP Mix	2 µL
RevertAid M-MuLV RT (200 U/µL)	1 µL
Total volume	20 µL

4. Mix gently and centrifuge briefly.
5. For $oligo(dT)_{15}$ or gene-specific primed cDNA synthesis, incubate for 60 min at 42°C. For random hexamer primed synthesis, incubate for 5 min at 25°C followed by 60 min at 42°C. **Note.** For GC-rich RNA templates the reaction temperature can be increased up to 45°C.
6. Terminate the reaction by heating at 70°C for 5 min.

The reverse transcription reaction product can be directly used in PCR applications or stored at -20°C for less than one week. For longer storage, -70°C is recommended