THE EFFECTS OF CAFFEINE AND TAURINE ON NEURON MORPHOLOGY

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Abstract
Caffeine and taurine are common ingredients listed on the nutritional label of energy drinks. However, in many drinks, these compounds are marketed as a "specialized blends." The consumption of energy drinks has increased in recent years, yet there is little research on neural morphology and the dosages of ingredients in these "specialized blends." The present study investigated the relationship of varying dosages of caffeine and taurine on primary rat cortical neurons by measuring the neuronal outgrowth of primary, secondary, and tertiary neurites within each treatment and concentration condition. This evaluation included seven treatments (excluding the control group) of both caffeine (100 μM -1mM) and taurine (50 μM -2mM), cultured over two weeks. Neurons were imaged using phase-contrast microscopy and analyzed using NeuronJ. A One-way ANOVA and Factorial ANOVA were employed. Data collected was used to establish a dose-response curve for each agent. Caffeine displayed a biphasic curve inferring two mechanisms working on neurite regulation and taurine displayed a U-shaped curve. An interaction between neurite type and concentration on neurite outgrowth was apparent [$F(12, 5901) = 3.34$, $p = .005$, $\eta^2 = .007$], suggesting concentration impacts the frequency of the types of neurons, which ultimately contributes to overall neuron length. Furthermore, the main effects for treatment ($p < 0.001$), concentration ($p < 0.001$), and neurite type ($p < 0.001$) on neurite outgrowth were revealed and will be discussed.

Keywords: caffeine, taurine, dose-response curve, neurite outgrowth

Introduction

The market for energy drinks is predicted to grow by 61 million by 2021 (Research and Markets, 2015). They are used ubiquitously in college and found in most vending machines on campus. In addition to their convenience, they are a popular tool to increase efficiency in completing day to day tasks. A typical energy drink usually contains 1g of taurine and 80 mg of caffeine, other drinks offer "specialized blends," in which the exact amount of ingredient is not disclosed. In adolescent brains, consuming high doses of caffeine induces indisposed effects; including indigestion (Curran & Marczinski, 2017) and influencing neuronal outgrowth (Connolly & Kingsbury, 2010). Neuron morphology is important because it provides insight into neural functions, connectivity, and integration as well as development. Previous studies investigated the effects of caffeine (Connolly & Kingsbury, 2010; Fazeli et al., 2017; Juárez-Méndez, et al., 2006) and taurine (Li et al., 2016; Wu & Prentice, 2010) independently; however, few studies have investigated caffeine and taurine in conjunction with one another (Santha et al., 2013; Schaffer et al., 2014) and even fewer studies establish a dose-response for caffeine or taurine in rat cortical neurons. Thus, we intend to establish a dose-response curve for neurite growth after exposure to caffeine and taurine and to predict how they affect neuronal outgrowth.

In the current literature, there are conflicting reports of taurine and caffeine effects, though it should be considered that none of the studies were replicates and each varies in methods and materials. The studies presented vary in the type of neuron (i.e. cortical rat neurons, neuroepithelial stem cells, age) and experimental methodology (i.e. immunohistochemistry, western-blot, PCR). Nonetheless, this acknowledges the need to conduct more studies to corroborate evidence of the neuronal morphology effects of caffeine and taurine.

Dose-response curve

Many agents have shown to enhance and regulate neurite outgrowth such as melanocortin's based on dose-response curves (Calabrese, 2008). Response curves are used to quantify the concentration of a chemical against its output, and useful to determine optimal ranges to manipulate concentrations to produce the desired effect. One major limitation in morphology literature is the lack of dose-response data (Curran & Marczinski, 2017). There are dose-response curves related to caffeine (Del Coso et al., 2012), but not in the context of rat neurons; the state of scientific understanding is the same for taurine (Nusetti, et al., 2005). In contrast to independent studies, an integrated investigation was completed and demonstrated a synergistic effect between caffeine and taurine across dose-response curves, but in the context of reduced platelet aggregates and hemostatic functioning (Santha et al., 2013). We anticipate the same will be portrayed in neurites; however, a foundation must be made to answer this overarching question by establishing both curves.

Caffeine and neuronal outgrowth

Caffeine, or *1,3,7-Trimethylpurine-2,6-dione*, has multiple neuroactive roles, including antagonism of adenosine receptors via blockade of A1 and A2A receptors and regulation of dopaminergic release. In addition, caffeine has been found to regulate neurite growth in response to concentrations less than 10 μM that stimulate neural CREB gene expression. In turn, activating expression of *BDNF* and IEGs, which promote neuron survival outgrowth and synaptic plasticity *in vitro* (Connolly & Kingsbury, 2010) and this mechanism suspected to explain why caffeine treatment groups (50 mg/kg) displayed longer dendrites *in vivo* than the control with no caffeine (Juárez-Méndez, et al., 2006). Concentrations of caffeine of 40 mM show decreased outgrowth, caused by Ca2+ dependent cone deterioration in neurons, though it should be noted this finding was collected from a neuroepithelial stem cell culture (Bandtlow, et al., 1993). The most recent study suggests medium doses of caffeine enhance outgrowth, while high >100 μM or low dosages <10 μM suppress outgrowth; more broadly, the cell's response to stimulus is dependent on the concentration, elapsed time of exposure (Yu et al., 2017), and age of neuron *in vitro* or *in vivo* the treatment is administered (Tchekalarova et al., 2014). In addition, caffeine was found to antagonize GABAA receptors which may increase physiological activity that could perhaps increase neurite outgrowth (Lopez, et al, 1989), by influencing the neurons to fire and wire together.

Taurine and neuronal outgrowth

Taurine, or *2-aminoethanesulfonic acid*, is a conditionally essential amino acid serving many functions in the central nervous system including acting as a neurotransmitter, regulating calcium homeostasis, and neuroprotective activity (Wu & Prentice, 2010). Excess taurine is excreted from the kidney's, but the most

taurine comes from meat and fish. A deficiency in taurine may lead to developmental abnormalities and severe eye impairments. Taurine is copious in the brain and structurally similar to GABA, or *4-aminobutanoic acid,* a neurotransmitter responsible for inhibiting action potentials by allowing Cl- to leak from the inner membrane, and has been widely accepted since the 1960's. Taurine specifically binds to GABAA receptors inhibiting the binding of GABA and showing hyperpolarizing effects but fails to affect GABA sensitivity and ultimately does not mediate response (Olmo, et al, 2000).

The effects of taurine concentration on neuronal outgrowth consistently demonstrate increased dendrite length in lower concentrations moderately promote neurite length (100 μM) while high concentrations (2.7 mM) will decrease neurite length and number (Shivaraj et al., 2012; Nusetti, et al., 2005). A dose-response curve was not found for taurine in the context of neurite length, but showed a sigmoidal curve for taurine and was just above baseline; the EC50 was 1.5mM (Schmieden, et al, 1992). The ranges of doses tested in the literature helped inform the exact range of doses that should be tested in this experiment.

Method

Gibco Cell Culture Cryopreserved Rat Cortical Neurons were obtained from Thermo Fischer Scientific (Cat. No. A1084001) and cultured in 7mL Falcon™ Standard Tissue Culture Dishes (Fisher Scientific; Cat. No. 353002) with poly-D-lysine (Sigma-Aldrich; Cat. No. P6407), laminin (ThermoFisher Scientific; Cat. No. 23017015), and phosphate-buffered saline (ThermoFisher Scientific; Cat. No. 10010023) for 20-40 minutes and left in the freezer for two days. Cells were cultured with 2ml of control medium. Four hours later the medium was changed to pull off any DSMO's. The cells were fed at 37° C with a medium containing 4% Fetal Bovine Serum (ThermoFisher Scientific; Cat. No. 10438018), Pen-strep (ThermoFisher Scientific; Cat. No. 15140122), B-27 (ThermoFisher Scientific; Cat. No. 17504044), Glutamax or L-glutamine (ThermoFisher Scientific; Cat. No. 25030081), and Neurobasal (ThermoFisher Scientific; Cat. No. 21103049) every four days for 2 weeks. Agents were not introduced until after two or three regular feedings with their respective working solutions depending on treatment. For a detailed explanation of Method, see reference (Pemberton, K., et al, 2018).

Trial one includes caffeine treatment: cells were suspended with a density of 191 cells/mL/mm^2 per culture dish. Working solutions were made to create the mediums containing caffeine concentrations of 5 μM, 10 μM, 25 μM, 50 μM, 100 μM, 500 μM, 1000 μM. Caffeine (Sigma Aldrich; Cat. No. C0750) treatment was not introduced until feeding 3. Trial two includes taurine treatment: Cells were suspended with a density of 234.8 cells/mL/mm^2. Working solutions were made to create taurine concentrations of 50 μM, 100 μM, 250 μM, 500 μM, 750 μM, 1 mM, 1.5mM, and 2mM. Taurine (Sigma Aldrich; Cat. No. T0625) treatment was introduced after feeding 2.

Neurons grown were fixed with Paraformaldehyde and rinsed twice with Phosphate Buffer Solution and then mounted with Mounting Medium (Sigma Aldrich; Cat. No. C9368). Fixed cells were imaged using phase contrast under a Leica microscope and analyzed by *ImageJ,* using the Fiji image package *NeuronJ*. Ten photos from each concentration were calculated. Two ANOVA tests will be conducted, a one-

way and a factorial, as well as a dose-response curve for each treatment. The statistics will be computed in *SPSS* to reveal any significant differences in morphology Figures 1 and 2 and *Prism* for Figure 3.

Results

One-Way ANOVA was conducted to test the relationship between concentrations and the control group on neurite length. The treatment concentrations range from 5 μM to 1 mM for caffeine, and 50 μM to 2mM for taurine with a total of 7 conditions for each treatment excluding the control. Caffeine revealed a significant difference in neurite length between the control and the various concentrations [$F(7, 3378) = 39.54$, $p<0.001$]. Taurine revealed there was a significant difference in neurite length between the control and the various concentrations [$F(7, 3206) = 6.424$, $p<0.001$]. Levene's test indicated homogeneity for both treatments ($p<0.001$), so post hoc test Tukey HSD was employed. In caffeine, Tukey (Table 3) showed an increase in neurite length in all concentrations in comparison with the control ($p<0.001$), except in 25 ($p=0.960$). In taurine, Tukey (Table 4) showed an increase in neurite length for half of the taurine concentrations (50 μM, 100 μM, 500 μM, 2mM) with respect to the control, and the other half showed no difference in neurite length (750 μM, 1 mM, and 1.5 mM). The means and standard deviations for the various caffeine and taurine concentrations, and the F values in Table 1 and Table 2 respectively. Tables for One-way ANOVA for caffeine and taurine not shown.

Table 1

Caffeine: Descriptive Table with F-value

[Caffeine] μM	M	SD	F
			39.54*
0	47.67	31.74	
5	75.91	50.43	
10	60.61	41.52	
25	50.69	40.23	
50	79.37	60.07	
100	89.60	59.58	
500	80.43	81.70	
1000	74.58	53.67	

Note: * Indicates a significance level of p<.001; [] indicate the concentration of treatment; M= Mean Length in μm; SD= Standard Deviation; F= F-value

Table 2

Taurine: Descriptive Table with F-value

[Taurine] μM	Mean Length	SD	F
			6.424* .000
0	47.67	31.74	
50	61.34	42.69	
100	59.55	44.44	
500	56.30	47.58	
750	50.87	40.10	
1000	50.72	38.14	
1500	55.95	45.08	
2000	56.31	34.91	

Note: * Indicates a significance level of p<.001; [] indicate the concentration of treatment; M= Mean Length in μm; SD= Standard Deviation; F= F-value

Table 3

Tukey HSD on Dependent Variable: Caffeine's Neurite Length (um)

CONTROL	[Caffeine] μM	Mean Difference	Std. Error	Sig. (P)
0.00	5.00	-28.24*	3.08	.000
	10.00	-12.94*	2.85	.000
	25.00	-3.02	2.78	.960
	50.00	-31.70*	4.03	.000
	100.00	-41.93*	3.67	.000
	500.00	-32.76	3.56	.000
	1000.00	-26.91*	3.20	.000

Note: * Indicates the mean difference is significant at the 0.05 level compared to the control. All values rounded to two decimal places except for the Sig. (P).

Table 4
Tukey HSD on Dependent Variable: Taurine's Length (um)

CONTROL	[Taurine] µM	Mean Difference	Std. Error	Sig. (P)
0.00	50.00	-13.68*	2.51	.000
	100.00	-11.83*	2.66	.000
	500.00	-8.63*	2.56	.017
	750.00	-3.12	2.50	.917
	1000.00	-3.16	2.54	.918
	1500.00	-8.28	3.04	.114
	2000.00	-8.64*	2.74	.035

Note: * Indicates the mean difference is significant at the 0.05 level compared to the control. All numbers were rounded to two decimal places except Sig. (P).

A Factorial ANOVA was conducted to evaluate the effects of treatment condition caffeine and taurine, seven concentrations of each treatment plus a control, and the type of neurites (primary, secondary, or tertiary) on neurite length. The ANOVA indicated a main effect for treatment [$F(1, 5901) = 205.27$, $p < 0.001$, $\eta^2 = .034$] , concentration [$F(9, 5901) = 3.670$, $p < 0.001$, $\eta^2 = .006$], and type [$F(2, 5901) = 22.70$, $p = .000$, $\eta^2 = .008$].The treatments main effect signifies caffeine (M=63.07, SE=3.06) has the greatest neurite length increase than taurine (M=55.86, SE=.93) compared to the control (M=43.10. SE=3.08). Although, taurine still shows longer neurites than the control with a significance between all treatments p<.001. The concentrations' main effect on length is not consistent, so we will rely on the significant interaction to give us more insight. The types main effect shows primary neurites (M=65.39, SE=.687) are longer than secondary (M=56.50, SE=2.02) and tertiary (M=52.69, SE=8.86) neurites overall for both treatments. No other significance was found between the other types of neurites overall. Individually, however, taurine and caffeine only showed a significant difference between primary and secondary. Further, a significant interaction was found between concentration and neurite type [$F(12, 5901) = 3.34$, $p = .005$, $\eta^2 = .007$], on neurite length. Factorial ANOVA results shown in Table 5.

Table 5
Factorial ANOVA

Source	df	Mean Square	F	Sig. (P)	Partial Eta Squared
Concentration	9	7811.04	3.67	.000	.006
Treatment	1	436887.95	205.27	.000	.034
Neurite Type	2	48291.69	22.69	.000	.008
Concentration * Neurite Type	18	7114.22	3.34	.005	.007
Error	5901	2128.41			
Total	5931				
Corrected Total	5930				

Table 5: The table above shows significant main effects and an interaction. All values were rounded to two decimal places except Sig. (P), and Partial Eta Squared.

A dose-response curve was established for both treatment conditions caffeine and taurine using mean lengths with respect to the control including error bars representing standard error. The dose-response curve for caffeine is a biphasic curve, and taurine's' curve displays a U-shaped curve. See Figures 1 and 2 respectively for curves. Non-significant results will not be displayed.

Figure 1

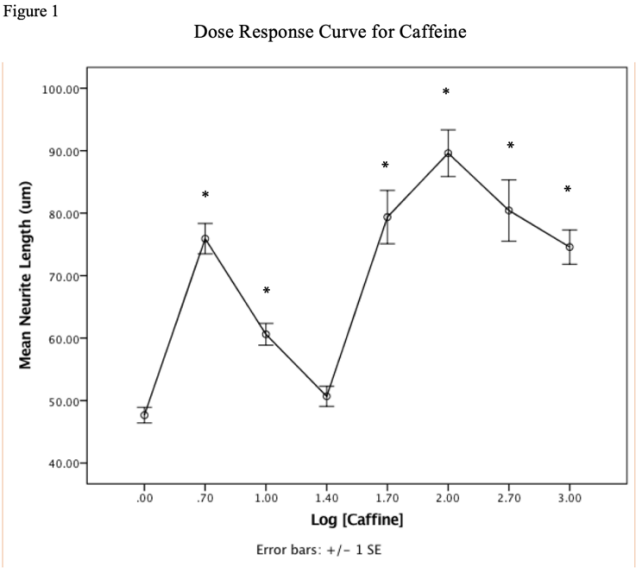

Figure 1: * Indicates a significance of p <0.001. Mean Neurite Length (µm) is plotted against caffeine concentrations which have been transformed into logs. This shows every concentration is significantly longer than the control except for 25 µM. Standard errors are reported.

Figure 2

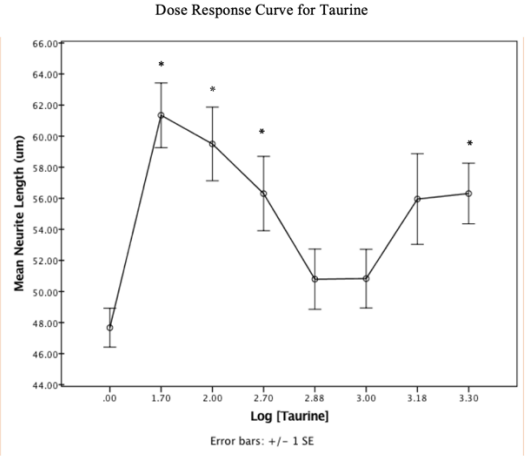

Figure 2: * Indicates a significance of p <0.001. Mean Neurite Length (μm) is plotted against taurine concentrations which have been transformed into logs. This shows every concentration is significantly longer than the control except for 750 μM, 1000 μM, and 1500 μM. Standard errors are reported.

Discussion

The main effect for treatment shows an overall 43.6 percent increase compared to the control for caffeine and an overall 29.6 percent increase for taurine. However, this does not give us insight into what concentrations we should be testing while keeping in mind the overall goal of this paper to build the foundation for further research. The main effect of concentration showed no obvious pattern as to what the difference was between each combination of concentrations except that most of each treatment's concentrations were significantly different from one another. The main effect for type based on the statistics main contribution was due to the difference in length between the primary and secondary neurons. Lastly, the interaction between concentration and type on neurite length suggests concentration impacts the frequency of types of neurons, which ultimately contributes to overall neuron length.

The results show caffeine exhibits longer neurites than the control which corroborates existing literature on neurite morphology (Connolly & Kingsbury, 2010). The caffeine dose-response displayed a biphasic curve. After another literature search, we found biphasic responses of caffeine present in other biological functions like heart rate and body temperature (Fredholm, et al, 2017), which may suggest multiple mechanisms affecting the response. Future studies may seek a timing dependent component with the inhibitory binding of caffeine with adenosine receptors, such as caffeine having different binding affinities for each adenosine A1, A2A and A3 receptor. Another possible mechanism to investigate is the density of adenosine receptors per neuron compared to each neurite length to give more insight into if the type of receptor is modulating the output response and how this is contributing the main effect of concentration.

Taurine's measured neurite length corroborates existing literature in the sense that they are not much longer compared to the control lengths. However, taurine's dose-response curve displays a U-shaped curve suggesting concentrations 750 μM, 1 mM, and 1.5 mM interfere with continued increased growth to baseline, and at those concentrations may suppress a growth regulator, or have a competitive binding mechanism for the GABAA receptor with GABA. Since GABAA receptors selectively allow Cl- ions to pass when activated it may induce an electrophysiological change that influences how often the neurons fire and possibly even how far they will grow towards another neuron and increase the neurite length. In another respect, taurine did not corroborate existing literature at concentration 50 μM and may allude to the fact of some sort of systematical error or contamination. Mistakenly, taurine concentration 250 μM was lost due to mounting a slide upside down during fixation. In result, this curve and should be tested again. Future studies should aim to establish and confirm a larger range of

concentrations for the continued effort to establish reproducible responses in neurites. Further, a deeper analysis should be pursued including but not limited to tracking genes transcriptions like *BDNF*, staining with immunofluorescence, and/or performing a Sholl Assay to test other aspects of morphology more generally.

The main effects found for each variable show we picked good doses to try to establish a foundation to help answer questions about these agents coexisting within a culture and the significance level them and especially between the concentrations indicate we picked appropriate dosages to address gaps in the literature that accurately captures overall behavior. However, this study fails to account for individual neuron differences. Nonetheless, neurites treated with caffeine grew longer in comparison to the taurine, but which agents' behavior would dominate the response if they were used as a treatment together? What if we took the lowest lengths concentrations of the taurine curve and paired them with the peaks of caffeine? What about combining the lowest caffeine concentration length with the peak of taurine? There is little literature addressing this question of combination; of both treatment and varying doses and examining common mechanisms such as serving as an agonist for the GABAA receptor. In retrospect, this experiment gives us a solid foundation to purposefully choose concentrations to produce meaningful tests in the future to examine formerly mentioned mechanisms. Figure 3 provides a transposed graph of both treatments to inform concentration combinations in future research.

Figure 3

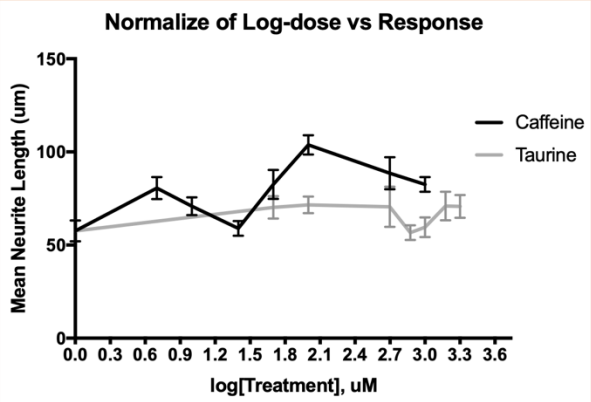

Figure 3: Displays log-dose for both treatments against mean neurite length (μm)and is a visual for future research to choose informed combinations of concentrations.

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