Dr. Andiara E. Freitas

TEL: +55 (48) 3721 5043
FAX: +55(48)3721 9672
andiaraef@gmail.com
andiara.freitas@posgrad.ufsc.br

Departamento de Bioquímica

Centro de Ciências Biológicas
Universidade Federal de Santa Catarina
Campus Universitário - Trindade
88040-900 Florianópolis, SC - Brazil

Committee for Aid and Education in Neurochemistry (CAEN)

CATEGORY 1B: Research supplies for use in the applicant's home laboratory

***Report** by Dr. Andiara Espíndola de Freitas*

Project Title: Agmatine potentiates the synaptogenic and antidepressant-like effects of subthreshold doses of ketamine

1) Overview

In December 2016 I was awarded a Research supplies for use in the applicant's home laboratory grant by the Committee for Aid and Education in Neurochemistry (Category 1B). I greatly appreciate the grant money that I received from the International Society for Neurochemistry because it covered important purchases which allowed me to develop the research project I lead. Importantly, this grant also covered some material used during the experiments performed by the Trainee students I supervise which gladly resulted in a manuscript submitted to the journal Neurochemistry International (IF 3.262).

During the course of this grant I had a pleasure to present my results in an international conference, namely the 2nd FALAN Congress, Argentina (2016). Additionally, I was a selected Speaker in the Young Members' Symposia at the 26th ISN-ESN Biennial Meeting, France (2017), and also a Selected Participant in the 14th ISN Advanced School of Neurochemistry "The energetic brain", France (2017). I have no doubt that the ISN support makes a great difference on my scientific training and aspirations, and all these achievements were essential for my future career in academia as an independent PI.

2) Background

Major depressive disorder (MDD) is a highly prevalent mental illness that affects approximately one in five people worldwide (Jorm et al., 2017). MDD causes a huge economic and social burden (World Health Organization, 2012) and it is estimated to become the second leading cause of disability by 2020 (World Health Organization, 2008).

In terms of pathophysiology, hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis is one of the most consistent biological discoveries in MDD since the 1960's. There is a wide consensus from preclinical and clinical studies that stress exposure is a key environmental factor in the etiology and progression of MDD (Gold et al., 1988, Lupien et al., 2009, Duman, 2014).

Studies demonstrate that a single, subanesthetic dose of ketamine, a glutamate N-methyl-D-aspartate (NMDA) receptor antagonist produces a rapid (within hours) and sustained antidepressant response effective in around 70% of treatment-resistant patients with MDD (Berman et al., 2000, Duman, 2014). The mechanism related to the

Dr. Andiara E. Freitas

TEL: +55 (48) 3721 5043
FAX: +55(48)3721 9672
andiaraef@gmail.com
andiara.freitas@posgrad.ufsc.br

Departamento de Bioquímica

Centro de Ciências Biológicas
Universidade Federal de Santa Catarina
Campus Universitário - Trindade
88040-900 Florianópolis, SC - Brazil

rapid antidepressant effects of ketamine involve activation of the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) and a subsequent increase in synaptogenesis (Duman, 2014). However, acute dissociative side effects and eventual fading of antidepressant responses limit widespread clinical use of ketamine. Several strategies have been proposed to address these limitations, ranging from the development of more selective NMDA receptor antagonists with reduced dissociative side effects to the addition of other antidepressant agents to limit the need for repeated ketamine dosing.

Agmatine is an endogenous polyamine synthesized by the enzyme arginine decarboxylase. It is a NMDA receptor antagonist and is considered as a neuromodulator in the brain (Piletz et al. 2013). As recently reviewed by Freitas et al. (2016), agmatine is emerging as a potential novel therapeutic strategy for MDD. Particularly, agmatine produces antidepressant-like effects through the activating of mTOR (Meylan et al., 2016; Neis et al., 2016). The potential synergistic effect of subthreshold doses of ketamine and agmatine is an innovative approach that might provide supporting evidence for the clinical use of agmatine as an adjuvant for MDD.

3) Hypothesis

I investigated the possibility that subthreshold concentrations of agmatine when combined with ketamine could afford protection against the cytotoxicity induced by corticosterone as well as the involvement of the mTOR/S6 kinase signaling pathway on such effect. I hypothesize that the co-administration of subthreshold concentrations of agmatine and ketamine will reduce the dissociative side effects of ketamine and improve its tolerance. Based on preliminary data from our laboratory, I would like to postulate that agmatine and ketamine share, for the most part, similar mechanisms of action.

4) Methods

4.1) Culture of HT22 cells

HT22 cells (a gift from Dr. Pamela Maher, Salk Institute, La Jolla, CA, U.S.A.) were cultured in Dulbecco's Modified Eagle Medium, supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, and 100µg/ml streptomycin. Cultures were seeded into petri dishes containing supplemented medium and kept at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. For assays, HT22 cells were subcultured in 96-well plates at a seeding density of 1×105 cells per well. Cells were treated with the drugs before confluence in DMEM with 10% FBS. Cells were used at passages below 13.

4.2) Cell viability assay

Mitochondrial function of HT22 cells was assessed by following the MTT reduction. Briefly, 50 µl of the MTT labeling reagent, at a final concentration of 0.5 mg/ml, was added to each well at the end of the treatment and the plate was placed in a humidified incubator at 37°C with 5% CO_2 and 95% air (v/v) for an additional 2 h-period. Active mitochondrial dehydrogenases catalyze the cleavage and reduction of the soluble yellow MTT dye into the insoluble purple formazan (Denizot and Lang, 1986). The insoluble formazan was dissolved by the addition of dimethyl sulfoxide, resulting in a colored compound whose optical density was spectrophotometrically

Dr. Andiara E. Freitas
TEL: +55 (48) 3721 5043
FAX: +55(48)3721 9672
andiaraef@gmail.com
andiara.freitas@posgrad.ufsc.br

Departamento de Bioquímica
Centro de Ciências Biológicas
Universidade Federal de Santa Catarina
Campus Universitário - Trindade
88040-900 Florianópolis, SC - Brazil

assayed at 540 nm. Results were compared to control samples, treated with vehicle (DMEM), to which 100% activity was attributed.

4.3) Propidium Iodide/Hoechst double staining for cell death

The cellular viability was visualized using nuclei staining with Hoechst 33342 and propidium iodide (PI), which are well-established and sensitive probes to determine apoptosis and necrosis. Hoechst 33342 is a blue-fluorescent probe staining all nuclei. In apoptotic cells, chromatin condensation occurs, and apoptotic cells can be identified as those with condensed and more intensely stained chromatin. PI is a red-fluorescent DNA-binding dye unable to cross the plasma membrane of living cells, but readily enters necrotic *(or late-stage apoptotic)* cells and stains their nuclei red. Briefly, at the end of the treatments, cells were double stained with Hoechst 33342 (1µg/mL), and PI (14 µg/mL) in a serum-free culture medium and the plate was placed in the dark for an additional 15-min period (Singh et al., 2016). PI/Hoechst fluorescence was analysed in a fluorescence inverted NIKON eclipse T2000-U microscope using filter sets, 488 nm excitation and 560 nm for PI and 353 nm excitation and 483 nm for Hoechst detection. Micrographs correspond to a representative experiment that was repeated three times with similar results. Images were taken using magnifications of 10×. Cell death (dead cells/total cells) was estimated by (PI/Hoechst) co-localization.

4.4) Western Blot

HT22 cells (5×10^6) were washed once with cold phosphate-buffered saline and lysed in 200 µl SDS-stopping solution (4% SDS, 2 mM EDTA, 500 mM Tris, pH 6.8), and were then boiled for 5 min. Lysates were centrifuged (10000 x g for 10 min, at 4ºC) to eliminate cellular debris, and the loading buffer (40% glycerol, 100 mM Tris-base, bromophenol blue, pH 6.8) in the ratio 25:100 (v/v) and β-mercaptoethanol (final concentration 8%) were added to the samples. Protein content was estimated by the method described by Peterson (1977) and concentration calculated by a standard curve with bovine serum albumin. Protein (30 µg) from these cell lysates was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitro-cellulose membranes using a semidry blotting apparatus (1.2 mA/cm2; 1.5 h). To verify transfer efficiency process, gels were stained with Coomassie blue (Coomassie blue R-250 0.1%, methanol 50%, acetic acid 7%) and membranes with 0.5% Ponceau S in 1% acetic acid. After this process, blots were incubated in a blocking solution (5% non-fat dry milk in Tris buffer saline solution (TBS) (10 mM Tris, 150 mM NaCl, pH 7.5) for 1 h at room temperature and targets were detected after overnight incubation (4ºC) with specific antibodies anti-phospho-Akt (1:3000, Sigma), anti-Akt (1:1000, Cell Signaling), anti-phospho-S6K (1:1000, Cell Signaling), anti-S6K (1:1000, Cell Signaling), anti-phospho-GSK-3β (1:5000, Cell Signaling), anti-GSK-3β (1:2000, Cell Signaling), anti-β catenin (1:2000, Santa Cruz), and anti-β actin (Santa Cruz, 1:2500) diluted in TBS with tween (TBS-T) that contained 2% BSA. Appropriate peroxidase-conjugated secondary antibodies at 1:2500 were used to detect proteins by enhanced chemiluminescence. All blocking and incubation steps were followed by three times washing (5 min) with TBS-T. β-actin immunocontent was evaluated as a protein load control. To detect phosphorylated and total forms of Akt, S6K, and GSK-3β in the same membrane, the immunocomplexes were stripped, as previously described (Freitas et al., 2014, Lopes et al., 2015). Briefly, membranes were washed once with deionized water (5 min), followed by incubation

Dr. Andiara E. Freitas
TEL: +55 (48) 3721 5043
FAX: +55(48)3721 9672
andiaraef@gmail.com
andiara.freitas@posgrad.ufsc.br

Departamento de Bioquímica
Centro de Ciências Biológicas
Universidade Federal de Santa Catarina
Campus Universitário - Trindade
88040-900 Florianópolis, SC - Brazil

with 0.2 M NaOH (5 min), washing with deionized water (5 min) and with TBS-T (10 min). The membranes stripped of immune complexes were blocked and followed the same steps described before. The optical density (O.D.) of the bands was quantified using Image Lab software®. The phosphorylation level of Akt, S6K, and GSK-3β were determined as a ratio of O.D. of phosphorylated band/O.D. of total band. The immunocontent of β catenin was determined by the relationship between the O.D of the β catenin band/O.D of β-actin band. Immunoblots correspond to a representative experiment that was repeated six times with similar results.

4.5) Statistical analysis

For cell viability assay, comparisons between experimental and control groups were performed by one or two-way ANOVA followed by Duncan's multiple range test, when appropriate. For western blot experiments, comparisons between groups were performed by Student's t test. $p<0.05$ was considered significant.

5) Results

5.1) Agmatine potentiates the neuroprotective effects of subthreshold concentrations of ketamine

After establishing the subthreshold concentrations of ketamine and agmatine that did not afford any neuroprotection, the ability of agmatine to potentiate the neuroprotective effect of a subthreshold concentration of ketamine on corticosterone-induced cell death was investigated. For this experimental approach, HT22 cells were pre-incubated for 24 h with subthreshold concentrations of ketamine (0.01 μM) and agmatine (0.001 μM) before the addition of corticosterone (50 μM), followed by a co-incubation for an additional 24-h period in the presence of corticosterone. In fact, agmatine was able to potentiate the protective effect of a subthreshold concentration of ketamine against corticosterone-induced cell death (**Fig. 1**). Of note, appropriate vehicle-treated groups were also assessed simultaneously, and the treatment of cells alone with agmatine or ketamine did not produce any significant neuroprotective effect (**Fig. 1**).

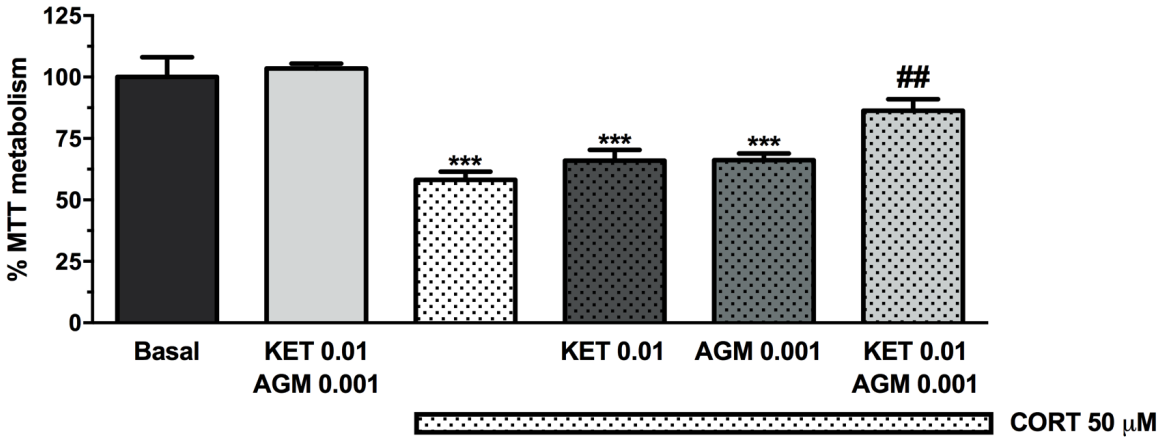

Fig. 1. Agmatine potentiates the neuroprotective effects of subthreshold concentrations of ketamine. HT22 cells were pre-incubated for 24 h with subthreshold concentrations of ketamine (0.01 μM) and agmatine (0.001 μM) before the addition of

Dr. Andiara E. Freitas

TEL: +55 (48) 3721 5043
FAX: +55(48)3721 9672
andiaraef@gmail.com
andiara.freitas@posgrad.ufsc.br

Departamento de Bioquímica

Centro de Ciências Biológicas
Universidade Federal de Santa Catarina
Campus Universitário - Trindade
88040-900 Florianópolis, SC - Brazil

corticosterone (50 μM), followed by a co-incubation for an additional 24-h period in the presence of corticosterone. Viability was measured as MTT reduction, and basal condition was considered as 100%. Each column represents the mean + S.E.M. of eight different cell batches. Statistical analysis was performed by two-way ANOVA, followed by Duncan's multiple range test ***p<0.001 as compared with the basal group; ##p<0.01, as compared with the corticosterone group.

5.2) Propidium Iodide/Hoechst cell death

In order to verify whether the combination of low-concentrations of agmatine and ketamine was able to abolish corticosterone-induced cell death, a PI/Hoechst double staining was performed. The experimental protocol consisted in pre-incubating HT22 cells with subthreshold concentrations of ketamine (0.01 μM) and agmatine (0.001 μM) before the addition of corticosterone (50 μM), followed by a co-incubation for an additional 24-h period in the presence of corticosterone. Appropriate vehicle-treated groups were also assessed simultaneously. Cell death was determined by PI/Hoechst co-localization (dead cells/total cells). The micrographs depicted in **Fig. 2** shows that corticosterone-treatment (panel **B**) caused a significant cell death on HT22 cells, as compared to the basal group (panel **A**) and verified as increased PI/Hoechst staining. Exposure of cells to subthreshold concentrations of agmatine or ketamine was not able to abrogate the corticosterone-induced cell death (panels **C** and **D**, respectively). Conversely, a subthreshold concentration of agmatine was able to potentiate the protective effects of a subthreshold concentrations of ketamine against corticosterone-induced cell death (panel **E**), since it abolished the increased PI/Hoechst staining observed in cells exposed to corticosterone.

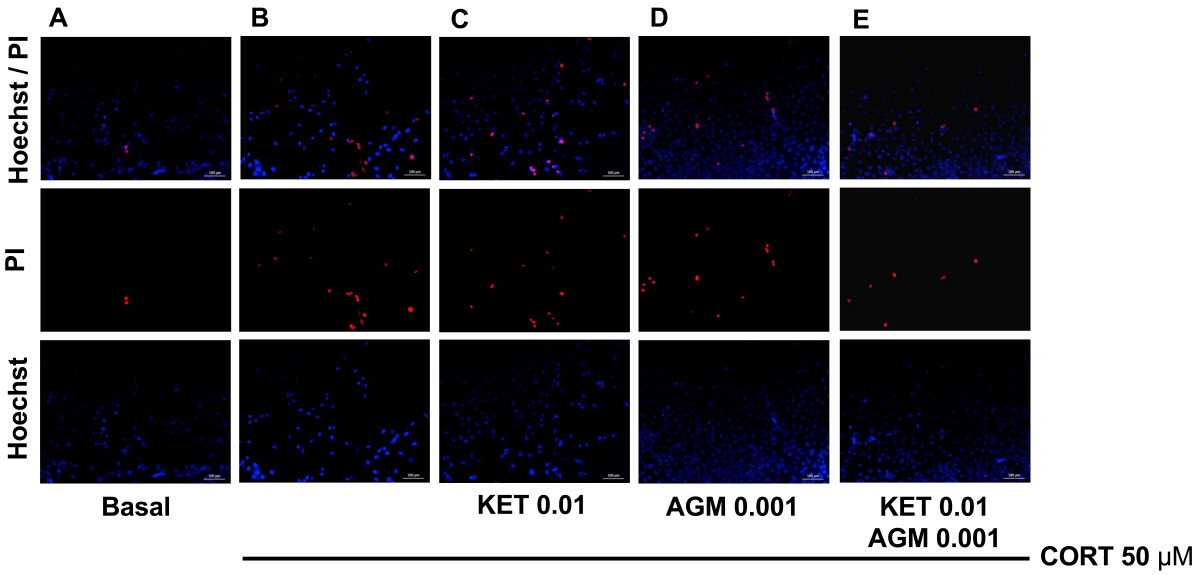

Fig. 2. Agmatine potentiates the protective effects of subthreshold concentrations of ketamine on corticosterone-induced cell death. Representative micrographs of **(A)** basal cells, **(B)** cells incubated with 50 μM corticosterone for 48 h, **(C)** cells incubated with 0.01 μM ketamine for 24 h followed by a co-incubation with 50 μM corticosterone for 24 h, **(D)** cells incubated with 0.001 μM agmatine for 24 h followed by a co-incubation with 50 μM corticosterone for 24 h, **(E)** cells co-incubated with 0.01 μM ketamine plus 0.001 μM agmatine for 24 h followed by a co-incubation with 50 μM corticosterone for 24 h. IP immunostaining: red; Hoechst counterstained: blue. Cell death estimated by PI/Hoechst (dead cells/total cells) co-

Dr. Andiara E. Freitas

TEL: +55 (48) 3721 5043
FAX: +55(48)3721 9672
andiaraef@gmail.com
andiara.freitas@posgrad.ufsc.br

Departamento de Bioquímica

Centro de Ciências Biológicas
Universidade Federal de Santa Catarina
Campus Universitário - Trindade
88040-900 Florianópolis, SC - Brazil

localization. Micrographs correspond to a representative experiment that was repeated three times with similar results Magnification, ×10.

5.3) Agmatine potentiates the effects of subthreshold concentrations of ketamine via mTOR/S6 kinase signaling pathway

We next decided to study the intracellular signaling pathway involved in the neuroprotective effects induced by the combination of subthreshold concentrations of agmatine and ketamine in HT22 cells. Since the mTOR/S6 kinase signaling is a key pathway implicated in the fast-action effects of ketamine (Duman et al., 2012); and agmatine is able to modulate mTOR/S6 kinase pathway (Neis et al., 2016), we selected this target to study.

Activation of PI3K/Akt has been reported to induce mTORC1 phosphorylation, and subsequent expression of synaptic proteins (PSD95 and synapsin 1) via S6K activation (Dwyer and Duman, 2013). In order to verify whether the combination of subthreshold concentrations of agmatine and ketamine is able to modulate mTOR/S6K signaling, HT22 cells were co-incubated with ketamine (0.01 μM) and agmatine (0.001 μM) for 24 h. Immediately after the co-incubations, HT22 cells were lysed and prepared for Western blot analyses. Appropriate vehicle-treated groups were also assessed simultaneously.

The results depicted in **Fig. 3** shows that a 24 h co-incubation with subthreshold concentrations of ketamine (0.01 μM) and agmatine (0.001 μM) was able to activate mTOR/S6 kinase pathway. A significant increase ($p<0.05$) in the phosphorylation levels of Akt (Ser^{473}) (panels **A** and **B**), and S6K (Thr^{389}) (panels **C** and **D**) was observed. Additionally, the co-incubation of cells with subthreshold concentrations of ketamine (0.01 μM) and agmatine (0.001) for 24 h induced a significant ($p<0.05$) increase in the immunocontent of PSD95 (panels **E** and **F**).

Dr. Andiara E. Freitas

TEL: +55 (48) 3721 5043
FAX: +55(48)3721 9672
andiaraef@gmail.com
andiara.freitas@posgrad.ufsc.br

Departamento de Bioquímica

Centro de Ciências Biológicas
Universidade Federal de Santa Catarina
Campus Universitário - Trindade
88040-900 Florianópolis, SC - Brazil

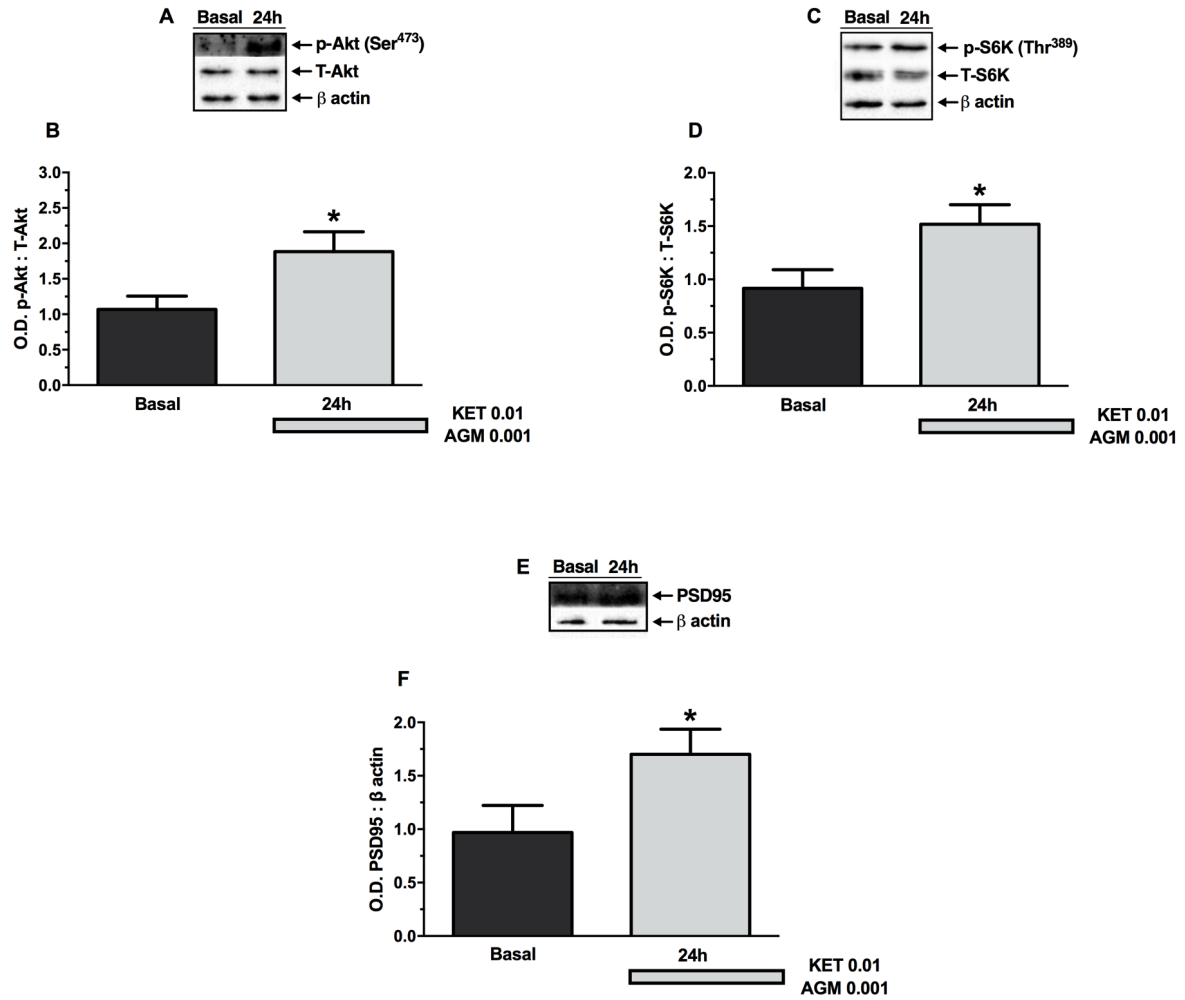

Fig. 3. Agmatine and ketamine synergist effect (24 h) is accompanied by Akt (Ser^{473}) (panels **A** and **B**) and S6K (Thr^{389}) (panels **C** and **D**) phosphorylation and PSD95 (panels **E** and **F**) immunocontent. HT22 cells were co-incubated with 0.01 μM ketamine plus 0.001 μM agmatine for 24 h. Panels **A**, **C**, and **E** show a representative western blot. Quantitative analyses are illustrated in panels **B**, **D**, and **F**. The data are expressed as ratio between phosphorylated (p-Akt) and total (T-Akt) forms, as ratio between phosphorylated (p-S6K) and total (T-S6K) forms, and as ratio between PSD95 content and β actin. Each column represents the mean + S.E.M. of six different cell batches. Statistical analysis was performed by Student's t test **p*<0.05 as compared with the basal group.

Finally, as depicted in **Fig. 4**, neither GSK-3β (Ser^{9}) phosphorylation (panels **A** and **B**) nor β-catenin immunocontent (panels **C** and **D**) were altered by the co-incubation with ketamine and agmatine for 24 h. Noteworthy, the treatment of cells with ketamine (0.01 μM) or agmatine (0.001 μM) *per se* (for 24 h) did not cause any significant effect on any protein analyzed (data not shown).

Dr. Andiara E. Freitas

TEL: +55 (48) 3721 5043
FAX: +55(48)3721 9672
andiaraef@gmail.com
andiara.freitas@posgrad.ufsc.br

Departamento de Bioquímica

Centro de Ciências Biológicas
Universidade Federal de Santa Catarina
Campus Universitário - Trindade
88040-900 Florianópolis, SC - Brazil

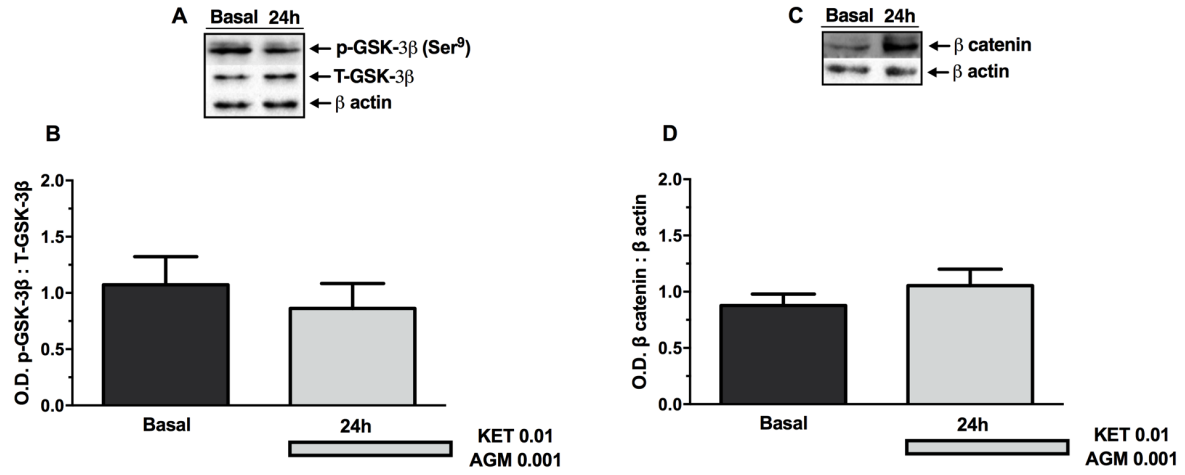

Fig. 4. Effect of agmatine and ketamine synergist treatment (24 h) on GSK-3β (Ser^9) (panels **A** and **B**) phosphorylation and β catenin (panels **C** and **D**) immunocontent. HT22 cells were co-incubated with 0.01 μM ketamine plus 0.001 μM agmatine for 24 h. Panels **A** and **C** show a representative western blot. Quantitative analyses are illustrated in panels **B** and **D**. The data are expressed as ratio between phosphorylated (p-GSK-3β) and total (T-GSK-3β) forms, and as ratio between β catenin content and β actin. Each column represents the mean + S.E.M. of six different cell batches. Statistical analysis was performed by Student's t test.

6) Conclusions

My results significantly extend literature data by indicating the agmatine's ability to potentiate the neuroprotective effect of subthreshold concentrations of ketamine against corticosterone cell death in a hippocampal neuronal cell line. I identified the induction of mTOR/S6 kinase signaling pathway as a critical target of ketamine and agmatine to afford neuroprotection and, most probably, such target is implicated in its antidepressant effects. The role of each of these proteins deserves further investigations to determine their direct relation to the augmenter antidepressant action of agmatine. However, the involvement of proteins which regulate synaptogenesis on the augmenter effect elicited by agmatine suggests that this neuromodulator should be further investigated as an adjuvant/augmenter drug to assist MDD therapy.

7) References

Berman, R. M., Cappiello, A., Anand, A., Oren, D. A., Heninger, G. R., Charney, D. S. and Krystal, J. H., 2000. Antidepressant effects of ketamine in depressed patients. Biol. Psychiatry, 47(4), 351–354.

Denizot, F. and Lang, R., 1986. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J. Immunol. Methods, 89(2), 271–277.

Duman, R. S., Li, N., Liu, R. J., Duric, V. and Aghajanian, G., 2012. Signaling pathways underlying the rapid antidepressant actions of ketamine. Neuropharmacology, 62(1), 35–41.

Duman, R. S., 2014. Neurobiology of stress, depression, and rapid acting antidepressants: remodeling synaptic connections. Depress. Anxiety, 31(4), 291–296.

Dr. Andiara E. Freitas

TEL: +55 (48) 3721 5043
FAX: +55(48)3721 9672
andiaraef@gmail.com
andiara.freitas@posgrad.ufsc.br

Departamento de Bioquímica

Centro de Ciências Biológicas
Universidade Federal de Santa Catarina
Campus Universitário - Trindade
88040-900 Florianópolis, SC - Brazil

Dwyer, J. M. and Duman, R. S., 2013. Activation of mTOR and synaptogenesis: role in the actions of rapid-acting antidepressants. Biol. Psychiatry, 73(12), 1189–1198.

Freitas, A. E., Bettio, L. E., Neis, V. B., Santos, D. B., Ribeiro, C. M., Rosa, P. B., Farina, M. and Rodrigues, A. L. S., 2014. Agmatine abolishes restraint stress-induced depressive-like behavior and hippocampal antioxidant imbalance in mice. Prog. Neuropsychopharmacol. Biol. Psychiatry, 50, 143–150.

Freitas, A. E., Neis, V. B. and Rodrigues, A. L. S., 2016. Agmatine, a potential novel therapeutic strategy for depression. Eur. Neuropsychopharmacol. 26(12), 1885–1899.

Gold, P. W., Goodwin, F. K. and Chrousos, G. P., 1988. Clinical and biochemical manifestations of depression. Relation to the neurobiology of stress (1). N. Engl. J. Med. 319(6), 348–353.

Jorm, A. F., Patten, S. B., Brugha, T. S. and Mojtabai, R., 2017. Has increased provision of treatment reduced the prevalence of common mental disorders? Review of the evidence from four countries. World Psychiatry, 16(1), 90–99.

Lopes, M. W., Lopes, S. C., Costa, A. P., Goncalves, F. M., Rieger, D. K., Peres, T. V., Eyng, H., Prediger, R. D., Diaz, A. P., Nunes, J. C., Walz, R. and Leal, R. B., 2015. Region-specific alterations of AMPA receptor phosphorylation and signaling pathways in the pilocarpine model of epilepsy. Neurochem. Int., 87, 22–33.

Lupien, S. J., McEwen, B. S., Gunnar, M. R. and Heim, C., 2009. Effects of stress throughout the lifespan on the brain, behaviour and cognition. Nat. Rev. Neurosci. 10(6), 434–445.

Meylan, E. M., Breuillaud, L., Seredenina, T., Magistretti, P. J., Halfon, O., Luthi-Carter, R. and Cardinaux, J. R., 2016. Involvement of the agmatinergic system in the depressive-like phenotype of the Crtc1 knockout mouse model of depression. Transl. Psychiatry, 6(7), e852.

Neis, V. B., Moretti, M., Bettio, L. E., Ribeiro, C. M., Rosa, P. B., Goncalves, F. M., Lopes, M. W., Leal, R. B. and Rodrigues, A. L. S., 2016b. Agmatine produces antidepressant-like effects by activating AMPA receptors and mTOR signaling. Eur. Neuropsychopharmacol. 26(6), 959–971.

Peterson, G. L., 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem., 83(2), 346–356.

Singh, R., Nawale, L., Sarkar, D. and Suresh, C. G., 2016. Two chitotriose-specific lectins show anti-angiogenesis, induces caspase-9-mediated apoptosis and early arrest of pancreatic tumor cell cycle. PLoS One, 11(1), e0146110.

World Health Organization, World Health Assembly, 2012. Global burden of mental disorders and the need for a comprehensive, coordinated response from health and social sectors at the country level: report by the Secretariat. World Health Organization (WHO), Geneva, Switzerland.

World Health Organization, 2008. The Global Burden of Disease: 2004 Update, World Health Organization (WHO), Geneva, Switzerland.

Dr. Andiara E. Freitas

TEL: +55 (48) 3721 5043
FAX: +55(48)3721 9672
andiaraef@gmail.com
andiara.freitas@posgrad.ufsc.br

Departamento de Bioquímica

Centro de Ciências Biológicas
Universidade Federal de Santa Catarina
Campus Universitário - Trindade
88040-900 Florianópolis, SC - Brazil

8) Financial Report:

Primary and Secondary antibodies	3,300
Albumin, Acrylamide, ECL reagent, Nitro-cellulose membrane	1,200
General laboratory consumables including drugs and tubes	500
Total ($)	5,000