NFκB1 and NFκB2 gene expression in the prefrontal cortex and hippocampus of early life stressed mice exposed to cocaine-induced conditioned place preference during adolescence

Rodrigo Orso[1*]; Kerstin Camile Creutzberg[1*]; Anderson Centeno-Silva[1]; Matheus Silva Carapeços[1]; Mateus Luz Levandowski[1,2]; Luis Eduardo Wearick-Silva[1,3]; Thiago Wendt Viola[1,3]; Rodrigo Grassi-Oliveira[1,2,3].

*Co-first Authorship

Email addresses: rodrigo_orso@hotmail.com; kerstin.creutzberg@acad.pucrs.br; anderson.centeno@acad.pucrs.br; mateuscarapecos@gmail.com; luzlevandowski@gmail.com; lewearick@gmail.com; thiago.viola@acad.pucrs.br; rodrigo.grassi@pucrs.br.

[1]Developmental Cognitive Neuroscience Lab (DCNL), Pontifical Catholic University of Rio Grande do Sul (PUCRS), Porto Alegre, RS, Brazil;

[2]Graduate Program in Psychology, Pontifical Catholic University of Rio Grande do Sul (PUCRS), Porto Alegre, RS, Brazil;

[3]Graduate Program in Pediatrics and Child Health, Pontifical Catholic University of Rio Grande do Sul (PUCRS), Porto Alegre, RS, Brazil.

CORRESPONDING AUTHOR:

Rodrigo Grassi-Oliveira

Avenida Ipiranga, 6681, prédio 11, sala 928 - Partenon, Porto Alegre, RS – Brazil – 90619-900

Tel.: +55 51 3320-3633 ext. 7740 – E-mail address: rodrigo.grassi@pucrs.br

ABSTRACT

Background: Neuro-immune pathways contribute to the onset and maintenance of cocaine-seeking behaviors, particularly through activation of NFκB signaling in the brain. However, the molecular mechanisms of this relationship are still not completely understood, especially considering the effects of early life stress, a major risk factor to cocaine addiction. The goal of this study was to investigate NFκB1 and NFκB2 gene expression in the prefrontal cortex (PFC) and hippocampus of mice exposed to early life stress and cocaine-induced conditioned place preference (CPP) within adolescence. Male BALB/c mice were randomly assigned to one of four groups: animal facility reared (AFR) with or without CPP training; maternal separation (MS) with our without CPP training. The MS animals were subjected to daily 3-h maternal separation from postnatal day (PND) 2 to 15. CPP was performed following three sequential phases: habituation (PND 34), conditioning (PND 35 to PND 44) and post-conditioning test (PND 45). Gene expression was determined by qPCR.

Findings: NFκB1 mRNA levels were decreased in the PFC of animals exposed to CPP compared to drug-naïve animals, while no difference was detected regarding rearing conditions. NFκB2 expression was upregulated in the PFC of animals exposed to CPP when compared to drug-naïve animals, particularly in animals exposed to MS with higher CPP scores. No significant effects were detected in the hippocampus.

Conclusions: Cortical NFκB2 up-regulation may be involved with the enhanced motivational salience for cocaine-paired cues observed in animals exposed to MS during adolescence.

Key words: Cocaine, Conditioned place preference, Early life stress, Maternal separation, NFkB mRNA.

INTRODUCTION

Early life stress can promote immune alterations across lifespan, including increased innate immunity/inflammation (1). Following chronic stress, up-regulation of an array of genes involved in pro-inflammatory pathways has been documented in both peripheral immune cell populations and brain-specific cells, such as astrocytes and microglia (2). Among possible pathways, Toll-like Receptors (TLRs) are the first line of defense against invading microorganisms and are responsible for triggering innate immune responses. TLRs expression are also modulated by stress exposure, inducing activation of the transcription factor nuclear factor-kappa B (NFkB) (3).

The NFκB regulates the expression of many pro-inflammatory cytokine genes (e.g., Interleukin-1, Interleukin-6, Tumor Necrosis Factor-Alpha) and it is also involved in the processes of synaptic plasticity and memory formation (4, 5). Therefore, chronic activation of this innate immune pathway can enhance inflammation in brain areas associated with mental disorders, and, consequently, contributing to the development of anxiety, depression and substance abuse disorder (6). In this sense, both clinical and pre-clinical investigations have pointed out a role for NFκB in the pathophysiology of cocaine drug addiction (7, 8). For instance, blocking the expression of NFκB in the nucleus accumbens (NAc) reduces the overall preference for cocaine use in mice, and decreases the number of dendritic spines in the NAc (8). Additionally, NFκB signaling in the frontal cortex was associated with cocaine-induced neurotoxicity and cocaine-induced memory impairments (9).

The NFκB family is composed of five proteins: relA (p65), NFκB1 (p50 and its precursor p105), NFκB2 (p52 and its precursor p100), c-Rel and RelB (10). Both p52 and p50 subunits do not have a transcription activation domain, so they need to interact with other factors to regulate

transcription (10). For this reason, RelB will interact with p100 and p52, and RelA and c-Rel will interact with p50 (11). Furthermore, both NFκB1 and NFκB2 have an important role in the regulation of immune and inflammatory responses, and the dysregulation in the expression of these genes can provoke the overproduction of pro-inflammatory cytokines, which are associated with several inflammatory disorders (12). Although NFκB1 and NFκB2 present similar structure and both are responsible for immune and inflammatory responses, they also have distinct functions. NFκB1 is mainly responsible for innate immune response, as well as controlling lymphocyte and macrophage function. On the other hand, NFκB2 has a main role in the organogenesis of peripheral lymphoid tissues and B-cell development, with a more pronounced function related to adaptive immunity (12).

Clinical and epidemiological evidence demonstrated that early life stress promotes higher vulnerability to substance use disorder development (13, 14). Therefore, using the Maternal Separation (MS) model of early life stress we have investigated the effects of neonatal chronic stress on cocaine-related phenotypes within adolescence, using the well-known Conditioning Place Preference (CPP) behavioral paradigm in mice (15). In this experiment stressed and control animals were exposed to cocaine and saline solution during 10 days of CPP training, and tested for cocaine/saline preference. We observed that adolescent male mice exposed to MS presented higher preference for cocaine paired cues and reduced cortical brain-derived neurotrophic factor (BDNF) gene expression, suggesting that exposure to early life stress can enhance the motivational salience for cocaine-paired cues, and that this effect may be partially mediated by BDNF signaling (Figure 1).

FIGURE 1 HERE

However, whether NFκB could be associated with the above-mentioned stress-induced CPP effect was not examined. Thus, the main goal of this study was to extend our previous work (15) by investigating NFκB1 and NFκB2 gene expression of animals exposed to early life stress that presented higher cocaine preference in the CPP, comparing it to control animals and animals not exposed to cocaine or CPP training. We focused this investigation in two important tissues for CPP, the prefrontal cortex (PFC) and hippocampus, given their role in conditioning acquisition, spatial clues recognition and memory consolidation, respectively (16, 17).

MATERIAL AND METHODS

Animals and Experiment Design

Male BALB/c mice were housed in standard mouse cages under a 12h /12h light-dark (lights on at 7 a.m.) cycle at 21 ± 1 C, humidity 55 ± 5% and ventilation. Food and water were available *ad libitum.* For this study, female mice were bred with a male (both at PND 60) for two days and the day of birth was considered PND 0. All experiments followed the NIH laboratory and animal care guidelines and were approved by the Ethics Committee on Animal Use (CEUA) of the Pontifical Catholic University of Rio Grande do Sul (PUCRS), Brazil under the registration #15/00473. Gene expression analyses were conducted in tissues harvested of animals exposed to MS and CPP and animal facility reared (AFR) animals exposed to CPP. In addition, in order to only investigate the effects of MS on transcriptional levels without the effects of CPP or drug administration, a group of drug-naïve animals were submitted to MS or AFR conditions, but were not exposed to any experimental conditions: CPP, drug administration, or handling.

Maternal Separation (MS)

MS is a robust early life stress protocol (18), where pups were separated daily from their dams for a period of 180 min (3 p.m. to 6 p.m.) from PND2 to PND14. Dams were transferred to another room and allocated in a new cage to avoid ultrasonic vocalization between dam and pups. The litter was kept together during the MS protocol in a new cage with clean bedding, and the temperature was controlled with a heating pad (33 ± 2 C) to maintain adequate body temperature. After the MS protocol both the litter and the dam were returned to the home cage. AFR group was left undisturbed until weaning, except for routine cage cleaning.

Cocaine-induced CPP

The Cocaine-induced CPP apparatus consisted two (15 x 15 cm) acrylic chambers divided by an acrylic barrier (3.2mm tick). Each chamber had a different visual cue printed on a white floor and background. One chamber had black lines and the other black circles. During the adolescence period three phases of the CPP were executed: Habituation (PND 34), conditioning (PND 35 to PND 44), and post-conditioning test (PND45). Mice spent 30 min exploring the chamber each day during all phases. During the habituation the animals had free access to both chambers, and the time in each of the chambers was recorded to verify pre-conditioning place preference. No animals exceeded the threshold of 70% time spent in only one chamber. During the conditioning procedure mice were assigned to receive cocaine in the chamber that they spent less time during habituation and saline in the other chamber. During the period of 10 days each animal received alternately cocaine and saline solution (5 days each). Half of the animals

received cocaine on days 1, 3, 5, 7, 9 and saline solution on days 2, 4, 6, 8, 10. The other half was exposed to the inverse pattern. After the last conditioning day, to test for cocaine-induced CPP the animals were allowed to explore both chambers with no drug/saline administration. To calculate the CPP score the time spent in seconds in the cocaine-paired chamber was subtracted by the time spent during the pre-test habituation phase. For more detailed information review Viola et al (2016). To perform the cocaine administration for the CPP a concentration of 20 mg/ml was obtained after dissolving Cocaine Hydrochloride in a sterile 0.9 % saline solution. A cocaine dose of 20 mg/kg (i.p.) was used since previous studies with BALB/c mice revealed that this cocaine concentration optimally induces place preference (19).

Brain sample preparation

Two hours after the CPP test at PND 45 euthanasia was performed by cervical dislocation without anesthesia in order to avoid pharmacological interference. After decapitation the brains were removed and stored overnight at 4 C in RNAlater (Ambion) solution (1 ml). All areas of the PFC were dissected by freehand technique without the olfactory bulb. After storing the samples at -80 C the hemispheres were randomly alternated within the groups, so half of the samples were from the left hemisphere and half from the right hemisphere.

Transcript mRNA levels

Total RNA was extracted using QIAzol (Qiagen) according to the manufacturer's protocol and suspended in RNase-free water. The RNA concentration was quantified using Qubit RNA Broad Range Assay in a Qubit Fluorometer 2.0 (Life Technologies). 250 ng of RNA from each sample was transformed into cDNA using miScript II RT kit (Qiagen) for mRNA gene

expression. The resulting 20 ul of cDNA were diluted with 80 ul of RNase-free water to a total volume of 100ul and stored at -20 C. 1 ul of each cDNA sample was used for RT-qPCR reaction, executed in a StepOne PCR machine (Applied Biosystems), using miScript SYBR Green PCR Kit (Qiagen). Each reaction was run in duplicate, and the ΔΔCt method was used to calculate the relative fold change expression with the AFR drug-naïve group as a reference. The Primers: NFkB1 (QT00154091), NFkB2 (QT00129864) and GAPDH (QT01658692) were purchased from Qiagen.

Statistical analyses

All data are presented as group mean ± standard error of the mean, and tested for normality using the Shapiro-Wilk test. A two-way analysis of variance (ANOVA) followed by Bonferroni post-tests was used to investigate the difference in mRNA expression between groups. A P-value of < 0.05 was considered statistically significant. All statistical analyses were conducted using SPSS software v.20.0 (SPSS, Chicago, IL, USA) and the graphs were generated using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

Cocaine-induced CPP effects on NFκB mRNA levels

We observed a decrease in NFκB1 mRNA levels in the PFC in animals exposed to cocaine-CPP compared to drug-naïve animals showing a significant treatment effect ($F(3,24) = 4.38$, $p < 0.05$) (Figure 2A). No significant group effect was detected between AFR and MS animals. In the Hippocampus we did not observed significant difference when comparing

184 animals exposed to cocaine-CPP and drug naïve animals or MS and AFR groups ($F(3,24) = 1.21$,
185 n. s.) (Figure 2C).
186 Regarding NFκB2 mRNA levels in the PFC we identified higher levels in animals
187 exposed to cocaine-CPP when compared to drug-naïve animals demonstrating a significant
188 treatment effect ($F(3,24) = 33.11$, $p < 0.01$) (Figure 2B). When comparing the MS-CPP group to
189 the AFR drug-naïve group we observed statistically significant difference (Bonferroni, $p < 0.01$),
190 but this difference could not be observed when comparing AFR-CPP group to AFR drug-naïve
191 animals (Bonferroni, n. s.). No significant group effect between AFR and MS from the same
192 treatment group was detected. Moreover, there was no significant group or treatment effects on
193 NFκB2 mRNA levels in the hippocampus ($F(3,24) = 3.21$, n. s.).
194
195
196 FIGURE 2 HERE
197
198 **DISCUSSION**
199 The data presented here suggest a novel role for the NFκB1 and NFκB2 genes regarding
200 the acquisition of cocaine-induced CPP, showing dynamically opposite effects in their
201 transcriptional activity following CPP test. Interestingly, we suggest that NFκB2 up-regulation in
202 the PFC may be involved with the enhanced motivational salience for cocaine-paired cues
203 observed in animals exposed to MS; while NFκB1 down-regulation was associated with cocaine-
204 induced CPP effects in the cortex, but its expression was not affected by stress-related effects.
205 Our results are in agreement with a previous study that observed a decrease in NFκB1
206 protein levels in the frontal cortex, an affect associated with memory impairments in animals

treated with intraperitoneal cocaine injections of 15mg/kg during 20 days (9). Thus although we did not test memory performance, the downregulation in the expression of cortical NFκB1 observed in our experiments could be involved with cocaine-induced neuroplasticity and PFC-dependent cognitive functioning alterations. Such hypothesis is supported by evidence that activation of NFκB1 is necessary for the regulation of synaptic plasticity and memory formation (4), in addition to its role of regulating innate immune functioning.

On the other hand, the enhanced motivational salience for cocaine-paired cues observed in the animals exposed to early life stress could possibly be associated with altered NFκB2 gene expression. Evidence suggests that NFκB2 transcription is induced by the B-cell activating factor (BAFF) (20). Although we did not investigate the expression pattern of BAFF, it is possible to hypothesize that an increase in BAFF levels triggers the activation of the IκB kinase complex, which induces the processing of p100 to p52, both subunits of NFκB2. There is evidence indicating that exposure to cocaine can enhance the release of BAFF, which will activate the cascade and increase the levels of NFκB2 (21). Although NFκB2 function in the has been understudied, the cortical BAFF-NFκB2 signaling pathway potentially has an important role in regulating cue-induced drug craving and drug-seeking behavior, and altered functioning of this pathway could be involved with higher cocaine-induced CPP attributed to early maternal separation exposure. There is evidence suggesting that the acquisition of CPP involves histones alterations resulting in increased expression of several genes including NFκB2 (22). Here we presented for the first time the effects of early life stress on cortical NFκB2 gene expression after CPP. In this regard, there is one study that found a pattern of demethylation in the promoter region of the NFkB2 in a genome-wide DNA methylation analysis from umbilical cord blood of neonates exposed to prenatal anxiety or depression of their mothers (23).

Moreover, while NFκB1 is constantly expressed in cortical regions (http://mouse.brain-map.org/experiment/show/77454688), NFκB2 has a low expression in the brain cortex during basal conditions (http://mouse.brain-map.org/experiment/show/76098410). Thus, activation of this "silenced" gene in the PFC may be a potential mechanism for higher vulnerability to cocaine and other drugs of abuse following repeated stress due to increased neuroinflammatory signaling. In this sense, previous evidence have showed that NFκB2 was downregulated in methamphetamine low drinking animals but upregulated in methamphetamine high drinking animals in the NAc (24). This is relevant since the majority of NAc neurons receive significant glutamatergic inputs from the PFC, an effect involved in the transferring of information regarding drug-associated environmental stimuli (25). Thus, synaptic alterations between the PFC and the NAc modulate acquisition of CPP behavior, and NFκB2 up-regulation is another molecular marker involved in this circuitry.

In conclusion, our study indicates that exposure to cocaine-induced CPP is associated with reduced NFkB1 and increased NFκB2 gene expression in the PFC. This increase in NFκB2 levels after exposure to cocaine use is potentiated by the neonatal stress. Thus, this data raise the possibility that the pro-inflammatory effects of cocaine may be enhance the long lasting pro-inflammatory processes derived from early stress exposure, contributing to the effects of stress on higher vulnerability for cocaine addiction and drug-related behaviors during periadolescence. Therefore, it will be interesting in future studies to determine whether manipulation of NFκB2 signaling in the PFC could attenuate the effects of early life stress on cocaine-related phenotypes.

LIST OF ABBREVIATIONS

AFR – Animal Facility Reared

ANOVA – Analysis of Variance

BAFF – B-cell Activating Factor

BDNF – Brain-derived Neurotrophic Factor

CEUA – Ethics Committee on Animal Use

CPP – Conditioned Place Preference

MS – Maternal Separation

NAc – Nucleus Accumbens

NFkB – Nuclear Factor-kappa B

PCR – Polymerase Chain Reaction

PFC – Pre-Frontal Cortex

PND – Postnatal Day

PUCRS – Pontifical Catholic University of Rio Grande do Sul

TLRs – Toll-like Receptors

DECLARATIONS

Ethics approval and consent to participate

The experiments were conducted in accordance with the NIH laboratory animal care guidelines and approved by the Ethical Committee on the Use of Animals of the Pontifical Catholic University of Rio Grande do Sul, Brazil.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq): 306271/2014-1, 400850/2014-1, 454429/2014-2.

Author's contributions

Conceived and designed the experiments: TWV, RGO. Performed the experiments: TWV, RO, KCC, ACS, MSC, MLL, LEWS, RGO. Analyzed the data: TWV. Contributed reagents/materials/analysis tools: RGO. Wrote the paper: TWV, RO, KCC, ACS, MSC, MLL, LEWS, RGO.

Acknowledgements

We would like to thanks Dr. Lucas Araújo Azeredo for his support during data collection.

299 **REFERENCES**

300

301 1. Grassi-Oliveira R, Honeycutt JA, Holland FH, Ganguly P, Brenhouse HC. Cognitive
302 impairment effects of early life stress in adolescents can be predicted with early biomarkers:
303 Impacts of sex, experience, and cytokines. Psychoneuroendocrinology. 2016;71:19-30.
304 2. Burke NN, Fan CY, Trang T. Microglia in health and pain: impact of noxious early life
305 events. Exp Physiol. 2016;101(8):1003-21.
306 3. Liu J, Buisman-Pijlman F, Hutchinson MR. Toll-like receptor 4: innate immune regulator
307 of neuroimmune and neuroendocrine interactions in stress and major depressive disorder. Front
308 Neurosci. 2014;8:309.
309 4. Engelmann C, Haenold R. Transcriptional Control of Synaptic Plasticity by Transcription
310 Factor NF-kappaB. Neural Plast. 2016;2016:7027949.
311 5. Jarome TJ, Butler AA, Nichols JN, Pacheco NL, Lubin FD. NF-kappaB mediates
312 Gadd45beta expression and DNA demethylation in the hippocampus during fear memory
313 formation. Front Mol Neurosci. 2015;8:54.
314 6. Bekhbat M, Rowson SA, Neigh GN. Checks and balances: The glucocorticoid receptor
315 and NFkB in good times and bad. Front Neuroendocrinol. 2017.
316 7. Bannon MJ, Savonen CL, Hartley ZJ, Johnson MM, Schmidt CJ. Investigating the
317 potential influence of cause of death and cocaine levels on the differential expression of genes
318 associated with cocaine abuse. PLoS One. 2015;10(2):e0117580.
319 8. Russo SJ, Wilkinson MB, Mazei-Robison MS, Dietz DM, Maze I, Krishnan V, et al.
320 Nuclear factor kappa B signaling regulates neuronal morphology and cocaine reward. J Neurosci.
321 2009;29(11):3529-37.

9. Muriach M, Lopez-Pedrajas R, Barcia JM, Sanchez-Villarejo MV, Almansa I, Romero FJ. Cocaine causes memory and learning impairments in rats: involvement of nuclear factor kappa B and oxidative stress, and prevention by topiramate. J Neurochem. 2010;114(3):675-84.

10. Kranick S, Ekanayake V, Martinez V, Ameli R, Hallett M, Voon V. Psychopathology and psychogenic movement disorders. Mov Disord. 2011;26(10):1844-50.

11. Lang AE, Voon V. Psychogenic movement disorders: past developments, current status, and future directions. Mov Disord. 2011;26(6):1175-86.

12. Hinson VK, Haren WB. Psychogenic movement disorders. Lancet Neurol. 2006;5(8):695-700.

13. Viola TW, Tractenberg SG, Levandowski ML, Pezzi JC, Bauer ME, Teixeira AL, et al. Neurotrophic factors in women with crack cocaine dependence during early abstinence: the role of early life stress. J Psychiatry Neurosci. 2014;39(3):206-14.

14. Andersen SL, Teicher MH. Desperately driven and no brakes: developmental stress exposure and subsequent risk for substance abuse. Neurosci Biobehav Rev. 2009;33(4):516-24.

15. Viola TW, Wearick-Silva LE, De Azeredo LA, Centeno-Silva A, Murphy C, Marshall P, et al. Increased cocaine-induced conditioned place preference during periadolescence in maternally separated male BALB/c mice: the role of cortical BDNF, microRNA-212, and MeCP2. Psychopharmacology (Berl). 2016;233(17):3279-88.

16. Chen CL, Liu H, Guan X. Changes in microRNA expression profile in hippocampus during the acquisition and extinction of cocaine-induced conditioned place preference in rats. J Biomed Sci. 2013;20:96.

17. Munoz-Cuevas FJ, Athilingam J, Piscopo D, Wilbrecht L. Cocaine-induced structural plasticity in frontal cortex correlates with conditioned place preference. Nat Neurosci. 2013;16(10):1367-9.

18. Tractenberg SG, Levandowski ML, de Azeredo LA, Orso R, Roithmann LG, Hoffmann ES, et al. An overview of maternal separation effects on behavioural outcomes in mice: Evidence from a four-stage methodological systematic review. Neurosci Biobehav Rev. 2016;68:489-503.

19. Eisener-Dorman AF, Grabowski-Boase L, Tarantino LM. Cocaine locomotor activation, sensitization and place preference in six inbred strains of mice. Behav Brain Funct. 2011;7:29.

20. Beinke S, Ley SC. Functions of NF-kappaB1 and NF-kappaB2 in immune cell biology. Biochem J. 2004;382(Pt 2):393-409.

21. Lood C, Hughes GC. Neutrophil extracellular traps as a potential source of autoantigen in cocaine-associated autoimmunity. Rheumatology (Oxford). 2017;56(4):638-43.

22. Renthal W, Maze I, Krishnan V, Covington HE, 3rd, Xiao G, Kumar A, et al. Histone deacetylase 5 epigenetically controls behavioral adaptations to chronic emotional stimuli. Neuron. 2007;56(3):517-29.

23. Non AL, Binder AM, Kubzansky LD, Michels KB. Genome-wide DNA methylation in neonates exposed to maternal depression, anxiety, or SSRI medication during pregnancy. Epigenetics. 2014;9(7):964-72.

24. Wheeler JM, Reed C, Burkhart-Kasch S, Li N, Cunningham CL, Janowsky A, et al. Genetically correlated effects of selective breeding for high and low methamphetamine consumption. Genes Brain Behav. 2009;8(8):758-71.

25. Koya E, Hope BT. Cocaine and synaptic alterations in the nucleus accumbens. Biol Psychiatry. 2011;69(11):1013-4.

FIGURE TITLES AND LEGENDS

Title: Figure 1. Behavioral outcome of CPP protocol in control and stressed animals.

Legend: A) AFR group was left undisturbed until weaning, except for routine cage cleaning; MS group was exposed to daily maternal separation from PND2 to PND14 for a period of 180 minutes. B) CPP protocol consisted in 3 phases. Habituation: mice had free access to both chambers for 30 minutes. Conditioning: mice were injected alternately with cocaine (striped chamber) and saline solution (dotted chamber) during 10 days; each administration was performed in one chamber. And test: mice were allowed to explore both chambers with no drug/saline administration.

Title: Figure 2. NFkB1 and NFkB2 mRNA expression in the Hippocampus and Pre-frontal cortex.

Legend: A) Significant treatment effect of NFkB1 mRNA levels in the PFC ($p < 0.05$, in a two-way ANOVA). No significant group or interaction effect was detected. B) Significant treatment effect of NFkB2 mRNA levels in the PFC ($p < 0.01$, in a two-way ANOVA). Multiple comparison analyses revealed significant differences between MS-CPP group compared to AFR drug-naive group (Bonferroni, $p < 0.01$). No significant group or interaction effect was detected. C) No significant group or treatment effect on Nfkb1 mRNA levels in the hippocampus was observed. D) No significant group or treatment effect on Nfkb2 mRNA levels in the hippocampus was detected. AFR drug-naïve n = 7, MS drug-naïve n = 7, AFR-CPP n = 7 — samples collected from the animals with higher CPP scores, MS-CPP n = 7 — samples from the animals with higher CPP scores. Data expressed as mean ± SEM. Single asterisk represents p

389 value below 0.05 in statistical analyses. Double asterisks represent p value below 0.01 in
390 statistical analyses.