Author's response to reviews

Title: Nitric Oxide and Histone Deacetylases Modulate Cocaine-Induced Mu-Opioid Receptor Levels in PC12 Cells

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Author's response to reviews: see over
Dear Dr. Morrey

We thank Dr. Kowalczuk for her e-mail of July 10, 2012 containing the reviews of our manuscript and inviting us to submit a revised version. We would also like to thank the reviewers for the comments and suggestions made. They were very helpful and as a result, we have completed additional experiments and made a number of changes to the text and data presentation in the paper. The specific revisions made, including the locations in the revised manuscript where these changes can be found, are indicated below.

Editor’s Letter

As requested we have now included a Methods section in the abstract of the manuscript (page 2, line 5 of the revised manuscript) and have moved the Methods section in the main text of the manuscript to follow the background (beginning on page 5 of the revised manuscript).

Reviewer #1

1. We agree with the reviewer that the toxic effects of cocaine on PC12 cells are an important consideration. Often, studies examining cocaine-induced cell death in PC12 cells have used higher doses than those employed in the present study (see Gramage et al 2008 Eur. J. Pharmacol.; Lepsh et al 2009 Mol. Brain, Numa et al 2011, Eur. J. Pharmacol.), however it is still possible that cell viability was affected in our study. Therefore, as suggested, we examined cell viability following 72 h of 100 μM RIT and 500 μM SCT of cocaine by MTT assay. The methods for this assay have been added to the revised manuscript (page 6, lines 16 to 21) and the data, indicating that the doses of cocaine used in our experiments were not toxic, have been included in the text of the results (page 14, lines 8 to 13 of the revised manuscript).

2. We appreciate the reviewer’s suggestions for describing additional potential mechanisms, with respect to the role of NF-κB in modulating opioid receptor levels and the relationships between NO, CREB and c-fos. Additional text and reference have been added to the discussion linking NF-κB to cocaine treatment and MOR expression (page 19 lines 6 to 9 of the revised manuscript). We have also added additional text to the discussion of the revised manuscript discussing the role of cocaine-induced CREB phosphorylation on c-fos expression (page 19, lines 3 to 6) and the links between NO and CREB (page 19, lines 12 to 15). As suggested, we have also added text to the discussion of the revised manuscript that implicates the D1 receptor in the regulation of NF-κB and potentially, NO production (page 21, lines 15 to 24). We also agree with the reviewer that the anti-inflammatory action of curcumin should be discussed and have therefore addressed this in the discussion of the revised manuscript (page 20, lines 14 to 18).
3. The reviewer’s suggestion for testing dopamine receptor antagonists is excellent. We have discussed this as a potential future direction for our work. Since PC12 cells express D1, D2, D3 and D4 receptors (see Chiasson et al 2006, Neurotox. Res; Chu et al 2004, Mol. Brain; Lepsch et al 2009, Mol. Brain) and these receptors have been linked to cocaine action and MOR levels (Bergman et al 2012, Int. J. Neuropsychopharmacol; Gago et al 2007, J. Comp. Neurol.; Zhuo et al 2007, Synapse) the role of dopamine receptors in the cocaine-mediated regulation of MOR expression would involve several additional experiments with multiple dopamine receptor inhibitors. Although this study would contribute substantially toward understanding the mechanisms involved, at this time they are beyond the scope of the current investigations.

4. As suggested by the reviewer, the labelling of Figure 2 A has been modified to include cocaine and the treatment time.

5. As requested by the reviewer, the accession numbers for the genes used to prepare the primers has been included in the methods section (see page 9 of the revised manuscript).

6. As suggested by the reviewer an alternative blot has been selected to depict MOR protein in Figure 2B.

7. In response to the reviewer’s suggestion, the location of action of curcumin has also been added to Figure 5; L-NAME was also added.

Reviewer #2

Overall

1. We agree with the reviewer that the toxicity of the cocaine treatments in our study is important to consider and as described above, we have assessed cell viability by MTT assay. The results from this assay indicated the PC12 cells in our study were still viable following 72 h of 100 µM RIT and 500 µM SCT of cocaine. While it is still possible some cells were lost before this time point, this would not impact HDAC activity as the activity is expressed per mg/protein which would be directly proportional to the number of cells present in each treatment condition.

2. The reviewer correctly points out adrenal cells have limitations when it comes to extrapolating the data obtained to intact organisms. We have revised the discussion to comment on some of these limitations (page 22, lines 4 to 8 of the revised manuscript).
1. The reviewer raises the issue of data analysis and statistics with respect to western blot analysis. The density of protein bands is measured in arbitrary units and to account for between blot variability we have expressed the density of each individual band as a ratio of the total density of all of the bands on the blot of that specific protein, not other irrelevant bands on each blot. The same is done for the house-keeping protein and then the relative levels of one protein to the other are calculated. In the revised manuscript the description of this analysis has been clarified (page 11, lines 20 to 23 and page 12, lines 1 and 2).

2. In the revised manuscript, the relative ratio calculations for western blot relative ratios was clarified as described above. For quantitative PCR, formulas for the analysis were previously provided (see page 12) and the software used as well as relative expression information was added to the revised manuscript (page 10, lines 1 and 2).

3. As suggested by the reviewer, we have provided justification for the differences in timing for the various assays (HDAC, RNA and protein). The changes can be found on page 10, lines 21 to 23.

4. In response to the reviewer’s suggestion, we have modified Figure 4 to show statistically significant differences.

5. As the reviewer points out, c-fos mRNA expression can be quantified. Following quantitative PCR analysis of c-fos, a significant increase in mRNA levels was detected and these results and have been added as Figure 3 B. Text describing these results has been added to the manuscript (page 15, lines 24 and 25, page 16, line 1).

6. Additional text and references were added to the manuscript to explain the selection of curcumin (page 7, lines 4 to 6). L-NAME is widely used as a non-selective NOS inhibitor. In the text of the manuscript we refer to our previous work (reference 53 in the revised manuscript) in which we have used 20 mM L-NAME inhibit NOS activity and to modulate gene expression in PC12 cells.

We believe these changes to the text and results have strengthened our manuscript. We hope the improved manuscript will now meet with your and the reviewers’ approval and will be considered acceptable for publication in BMC Pharmacology and Toxicology.

Sincerely

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