Reply to “Reproducing Increased Dopamine with Infection To Evaluate the Role of Parasite-Encoded Tyrosine Hydroxylase Activity”

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In their letter, McConkey et al. (1) suggest that differences in study design may have led to different results in our study compared with those of previous reports. In an effort to better understand these differences, we welcome the opportunity to provide more insight into the rationale for our study design and the reasoning behind the interpretations we reached.

We stress at the outset that we had hoped to validate the previous findings that infection by Toxoplasma gondii leads to elevated dopamine production by dopaminergic cells in vitro and in chronically infected mice. In anticipation of such findings, we set out to disrupt the AAH2 gene, which had been implicated in this process by indirect data published previously by the McConkey group (2). Unfortunately, our studies failed to reproduce the original findings that infection increases dopamine production by using various parasite strains in different in vitro and in vivo models (3). In addition, review of the prior literature pertaining to the effects of T. gondii infection on elevated dopamine levels in vitro indicates that this pattern is highly variable (see points below).

Because of the study design, the difficulties encountered in reproducing the original findings were not apparent until we had already generated the knockout and complement strains and begun testing them in parallel. This approach was chosen intentionally as an unbiased method for testing the hypothesis without a priori assumptions. We do not agree with the statement that it is essential to prove that our parental strain induces dopamine prior to engaging in gene knockout studies. Rather, our findings call into question whether the original findings are robust or generally reproducible. Although the letter by McConkey et al. suggests that our failure to observed elevated dopamine levels might be due to technical differences, we do not find this a compelling argument, as detailed in the following point-by-point response.

(i) The authors claim that in their experiments, PC12 cells were kept competent to produce dopamine via attention to low passage number. We agree with the importance of this experimental detail. Our PC12 cells were expanded and frozen as low-passage-number stocks shortly after being received from ATCC. In all experiments, cells were maintained for only ~10 passages before being renewed from stock vials.

(ii) The authors claim that our bradyzoite treatment, where we used high-pH growth medium to incubate infected PC12 cells, could quench dopamine production in these PC12 cells. Indeed, that is what we observed, a 25-fold decrease in total dopamine content produced by PC12 cells cultured under alkaline conditions. However, the result that was of interest to us was whether bradyzoites contribute to dopamine production in infected PC12 cells, as proposed by Prandovzsky et al. (2). If this prediction is correct, it stands to reason that the result should still hold even if dopamine production is globally lower. The fact that we still did not see a difference in overall dopamine content despite demonstrable evidence of differentiated bradyzoite-containing vacuoles inside PC12 cells challenges this hypothesis (3).

(iii) The authors claim that their technique, soaking liberated tachyzoites in high-pH medium for 16 to 18 h and then infecting PC12 cells, differentiates the parasites into the bradyzoite stage without affecting the PC12 cells. We attempted to infect PC12 cells with bradyzoites that were liberated from high-pH-treated infected human foreskin fibroblast cells after 48 h, a time point when they stain positively for the Dolichos biflorus lectin (DBL), under conditions consistent with those of Prandovzsky et al. (2). However, at 48 h postinfection, the proportion of lectin-positive vacuoles was extremely low (~12%). We expect that this result came from parasites differentiating back into tachyzoites without continued pH stress to enforce the bradyzoite state. When we tried using high-pH treatment of extracellular parasites as reported by Prandovzsky et al. (2), we observed very poor viability, such that it was not possible to reliably infect PC12 cells. Hence, we modified the protocol to subject infected PC12 cells to high-pH medium, thereby inducing 100% DBL-positive vacuoles (i.e., bradyzoites). Under these conditions, we failed to see dopamine changes (3).

(iv) The authors claim that in the absence of viability data, it cannot be excluded that the 25-fold dopamine decrease in alkaline PC12 cells could be due to cell death. PC12 cells were counted by light microscopy before being harvested into perchloric acid buffer for high-performance liquid chromatography, and the level of dopamine per 10^5 cells was determined on the basis of visual identification of intact cells. If alkaline treatment led to increased mortality of PC12 cells, this would be corrected for by this visual analysis.

(v) The authors rightly point out that parasite strain differences might exist in the effects on dopamine and behavior and also point out that our strain, PruΔku80Δhxg, has not been previously demonstrated to induce elevated dopamine prior to gene knockout. However, PruΔku80Δhxg was not the only strain used in our study (3). We observed the same absence of dopamine change in vitro when using ME49 (unpublished data). In vivo, we demonstrated no change in brain dopamine not only in mice infected with PruΔku80Δhxg but also in mice infected with ME49 and the original C56 strain used by Stibbs (4). Thus, the failure to observe

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elevated dopamine is not limited to the Pru background used for genetic studies. The authors also suggest that the differences in our data reflect different mouse strains. Although we agree that infection of different mouse strains can lead to different outcomes, we failed to observe an increase in dopamine across a wide range of infection burdens caused by parasite strains C56 (low burden) and ME49 (high burden), and we failed to observe a correlation between cyst burdens and brain dopamine levels across this range (3). It is also worth pointing out that the range of dopamine levels that we observed in infected and noninfected mice was similar to that reported by Stibbs (4) and that the reason Stibbs was able to claim a statistically significant difference was an unusually small variance in the data. In summary, these findings suggest that if infection alters dopamine, this phenotype is not robust to strain or host differences and call into question the generality of previous claims.

(vi) The authors cite three studies in addition to the Stibbs study as evidence that Toxoplasma causes changes in dopamine neurotransmission. One is the authors’ own work, and the other two are studies by Gatkowska et al. (5) and Xiao et al. (6). In their study, Gatkowska et al. (5) examined changes in the ratios of neurotransmitters to their metabolites. Although their results and described differences in metabolite profiles of catecholamines are very compelling, they reported that these differences occur predominantly in acute infection and these ratios largely return to the baseline during chronic infection (5). Meanwhile, Xiao et al. (6) described dramatic differences in gene expression in the mouse brain upon chronic infection, especially genes related to cognition, olfaction, and dopamine receptors. However, they did not describe neurotransmitter levels. Taken collectively, these studies do not provide compelling support for the hypothesis that infection causes global changes in dopamine, although we acknowledge that in our study, we cannot rule out local changes that might be important for behavior differences.

(vii) The authors object that ToxoDB data show AAH expression in line with metabolic enzymes but lower than that of structural proteins and cite our results showing that AAH levels are greater than those of lactate dehydrogenase 2. We felt that this is a misunderstanding of our figure that displays the relative expression change from tachyzoite to bradyzoite (3). On an absolute level, in terms of raw cycle threshold (CT) values obtained by quantitative PCR, the 10-fold increase in AAH gene expression brought CT from ~28 to ~25, corresponding to tachyzoite-to-bradyzoite values. For comparison, actin ranged from ~20 to 21 constitutively. LDH2 went from ~24 to ~21 from tachyzoites to bradyzoites (also a 10-fold increase, as noted by the authors, but 16-fold higher absolute levels of expression than AAH genes), and BAG1 ranged from ~26 to ~19 from tachyzoites to bradyzoites. Therefore, the expression of the AAH genes when induced in bradyzoites is comparable to the expression level of BAG1 in tachyzoites, where it is considered to be off. Between all of the stages of the Toxoplasma life cycle, it is estimated that roughly 15 to 20% of its genes are switched off at any particular stage (7, 8). Indeed, across numerous sets of expression data in ToxoDB, the AAH genes rank between the ~8th and 20th percentiles in expression levels in tachyzoites and go up to the ~40th percentile (and the 60th in a minority of data sets) in bradyzoites. In comparison, LDH2 goes from the ~20th percentile in tachyzoites to the ~90th to the 100th percentile in bradyzoites. This is consistent with the expression of the parasite AAH genes being extremely low and modestly induced in bradyzoites. Notably, we observed similar expression levels and changes for AAH1 and AAH2 (3), which is also consistent with data in ToxoDB.

(viii) The authors point out that with two extremely homologous AAH genes, isolation of the effects of one copy is difficult and that we cannot rule out compensatory effects from the other gene. This is a valid objection and one that we acknowledge in our own paper. Indeed, we cannot rule out compensatory effects of the remaining gene masking the effects of the single knockout. However, what we observed was a complete absence of any parasite effect on dopamine at all, even in our wild-type strains. Additionally, the AAH1 gene being refractory to knockout is inconsistent with the genes having a role in manipulation of host neurotransmission. If this model were true and this were the sole function of AAH1, it would be unlikely for the gene to be essential to parasite growth and development. Rather, we think that the AAH genes, or at least AAH1, serve functions for the parasite other than the proposed function of manipulating host neurotransmission. Conditional-knockout studies are under way to try to untangle the function of AAH1 and shed some light on why this gene appears to be essential.

In summary, we feel that the available data do not support the conclusion that infection by T. gondii induces reliable changes in dopamine levels in cells or in infected animals, hence suggesting a different role for the AAH genes.

REFERENCES