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Supplemental Material to:

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Methylthioninium chloride (methylene blue) induces autophagy and attenuates tauopathy in vitro and in vivo

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Published data suggests that MB improves cognition[1] in a tau transgenic mouse model that has rapid and severe pathology development, cognitive impairment and neurodegeneration.[2] To assess whether MB treatment in our hands had a similar effect, we administered MB to the same line of tau mice as previously described. The route of administration was the same (in drinking water for 3 months) but the dose was doubled (from 10mg/kg/day to 20mg/kg/day) as the published study found that only mice with the highest brain levels of MB showed cognitive improvement. Mice at two stages of disease were tested – mice started on MB at 2.5 months of age have minimal pathology and very mild cognitive impairment whereas mice started at 5 months of age have overt pathology, degeneration and cognitive impairment. As effects on pathology, degeneration and cognition have been reported previously for MB in this line of mice[1], data presented only reflects our confirmation of the effects on cognition and phosphotau levels.

MB treatment improves cognitive performance Transgenic mice from the rTg4510 line and non-tg littermate controls were given either 20mg/kg per day of MB or water vehicle ($n = 9$ or 10 for each group). Mice were given four trials per day for six days. A maximum of sixty seconds was allowed for the mouse to find the platform. If the mouse did not find the platform after 60 seconds, it was placed on the platform for a further 30 seconds. Total distance travelled, escape latency and swim speed were recorded for each animal. Following the trial animals were towel dried and placed in a cage with additional towels until dry and then returned to the home cage. A probe trial was conducted on the seventh day and the time spent in each quadrant was recorded. Vehicle treated rTg4510 mice showed no significant improvement in the task after 6 days. However, a majority of the MB treated animals (7 out of 10) demonstrated significant improvement on the fifth and sixth day of testing ($p < 0.003$). When the groups were separated by sex, the effect was even more dramatic with treated males showing significant improvement relative to vehicle treated mice after three days. By this time, the level of performance was the same as non-tg animals (either MB or vehicle treated). rTg4510 females have a more pronounced phenotype than males at all ages[3] and in this study, they remained memory impaired for the duration of behavior testing. Male transgenic mice which began treatment at 5 months of age ($n = 4$) also showed significant reduction in latency to find the platform by day three of testing. Similar to the younger mice, aged females did not show a significant change.

MB reduces tau concentration in the brains of treated animals. Following three months of MB treatment, brains were collected and fractionated as described. Immunoblotting for total and phospho tau was carried out. Significant reductions in tau levels were observed whether the results were quantified together, or by sex ($p = 0.0002$). Similarly, tau phosphorylated at $ser^{202/205}$ was reduced in animals who received MB. However, when normalized to total tau levels there was no significant hypophosphorylation ($p = 0.12$). Interestingly, levels of $ser^{202/205}$ strongly correlated with performance in the water maze test. Average time to reach the platform on the last day of testing was plotted against the chemiluminescent signal obtained from immunoblotting which yielded a linear regression with an R^2 of 0.679 .

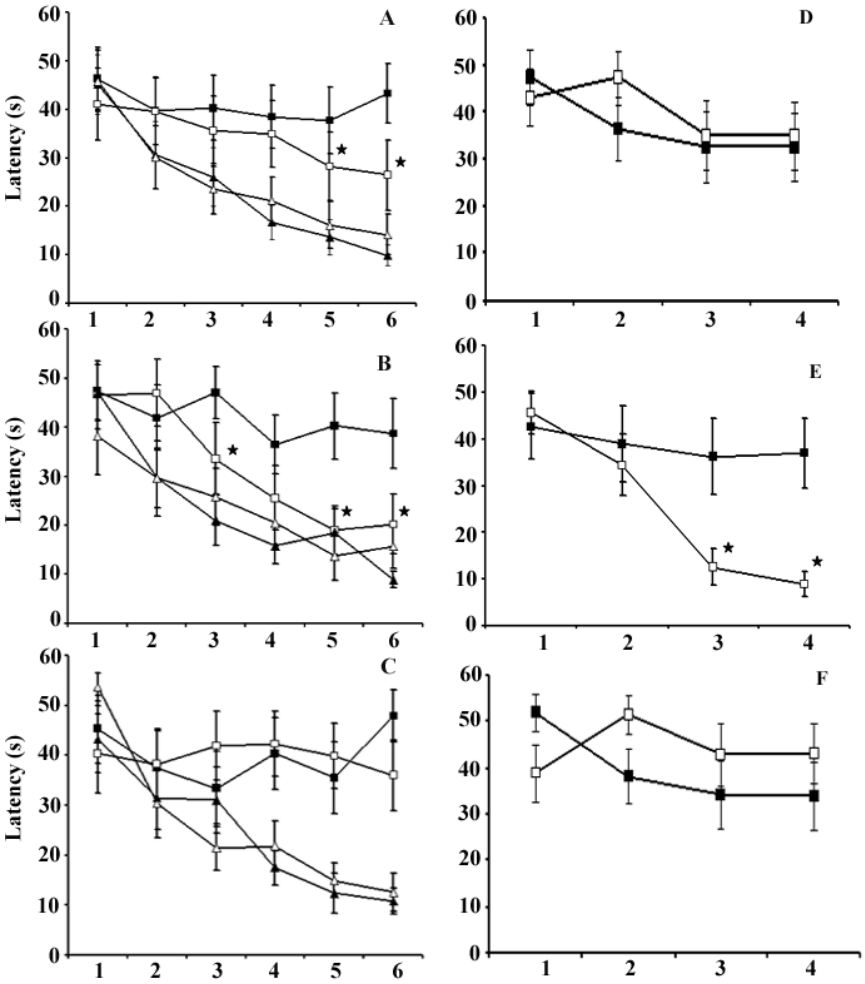

Figure 1. MB treatment improves cognitive performance. A. rTg4510 mice transgenic mice given 20 mg/kg MB for three months (□) showed significant improvement over water treated animals (■) on days five and six of testing ($p < 0.02$). No difference between treatment groups was observed for non-tg animals (black triangles for water vehicle and white triangles for MB treated animals) **B**. For males alone (n = 4 or 5 per group) significant reductions ($p < 0.05$) in escape latency were observed beginning on day three of testing in MB treated animals. No significant changes were observed between treatment groups in non-tg animals. **C.** Transgenic females (n = 5) receiving MB were not significantly improved relative to vehicle treated animals at any point in the study. **D.** rTg4510 mice aged 5 months were given MB or water vehicle as described. No significant change in latency was observed over four days. **E.** Males alone (n = 4) again showed significant improvement relative to control animals ($p < 0.01$). All values are the group average ± sem.

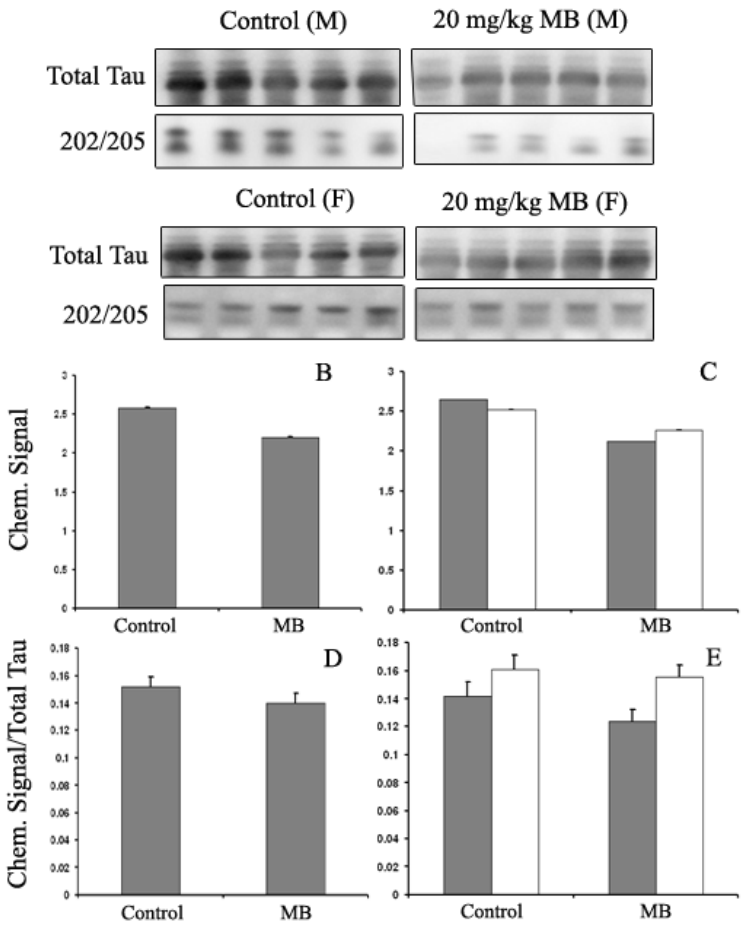

Figure 2. MB reduces total and phospho-tau. Following behavior testing, the cortex and hippocampus were fractionated as described above. **A.** Levels of total tau and tau phosphorylated at ser$^{202/205}$ were assayed. **B,C**. Both male and female rTg4510 mice treated with MB show significant reductions in total tau levels when quantified together (2.58 ± 0.0069 control, 2.20 ± 0.0053 treated) ($p = 0.0002$) or by sex (males grey bars, females white bars). **D, E.** Levels of tau phosphorylated at ser$^{202/205}$ were reduced to the same extent as total tau.

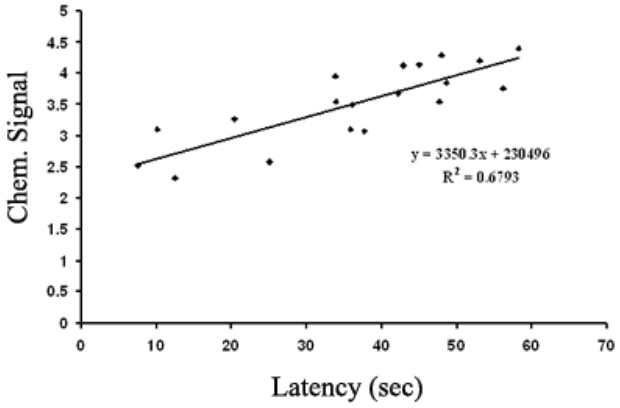

Figure 3. Phospho ser$^{202/205}$ correlates with escape latency. Immunoblotting for tau phosphorylated at ser$^{202/205}$ was performed and chemiluminescent signal was determined. Values were potted against average latency on day 5 of behavior testing. A linear regression yielded an R^2 of 0.679.

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