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Appendix E1

Study Design Explanation

There are no functional MR imaging and almost no pharmacologic studies on the use of methylene blue in humans. We considered a crossover design for the methylene blue trial but were concerned about potential carry-over effects between treatments. Specifically, unlike shorter-acting drugs such as caffeine, 1 month after a single oral dose of methylene blue, the treated subjects still demonstrated the behavioral effects of methylene blue, different than those treated with a placebo in a previous study (1). We could not confidently predict the amount of time methylene blue or its effects would last in the body, which is a problem if one-half of the subjects will first be on methylene blue. We would not know when to bring the subjects back for a second imaging examination. This setup also raises the possibility of losing subjects to follow-up. Another limitation is that methylene blue could alter baseline mood and affect subsequent image acquisitions. Additionally, one-half of the subjects would also be exposed to different learning effects. The performance on the second imaging examination is expected to be better because of learning effects, decreased anxiety toward the imager or drug, and potential paradigm recall. Another problem that is not encountered with most drugs is that 100% of the subjects who received methylene blue also reported colored urination, an expected finding; none of the placebo subjects reported this side effect. Therefore, subjects would be able to suspect whether they had received the methylene blue or placebo pills on the basis of the presence of urine coloration, which would also introduce bias and prevent participants from being blinded to

methylene blue treatment. In summary, our two-arm double-blinded methodology could be conducted as a one-visit study without many of the limitations imposed by a crossover design and by using a drug like methylene blue (rather than shorter-acting drugs, such as caffeine).

Sustained Attention (Psychomotor Vigilance Task Paradigm)

The psychomotor vigilance task is a standard task used to evaluate sustained attention (2). Subjects were shown a visual stimulus (a green cross) that signaled them to press a button as soon as a second stimulus (a red cross) randomly appeared on the screen after 2, 4, or 6 seconds (Fig 2b). The reaction time was provided to the subject after the button was pressed and was logged by the program. A corresponding rest period (a blank screen) followed, for a sum total block length of 14 seconds. Each session consisted of 30 blocks.

Before administration of methylene blue, the psychomotor vigilance task activated cortical and subcortical regions that were previously described as being activated in this task (3,4). It also produced deactivation in the medial prefrontal cortex, posterior cingulate cortex, precuneus, and bilateral inferior parietal cortex, a finding consistent with expected downregulation of the default-mode network that is generally deactivated by cognitive tasks (5,6).

Short-term Memory (Delayed Match-to-Sample Task Paradigm)

We used a delayed match-to-sample task that was previously used to successfully evaluate memory-enhancing effects in humans (Fig 3d) (7). During encoding, the subject was told to memorize a 6 × 6 grid pattern of yellow and red boxes. During the maintenance phase, the screen went blank, and the subject kept his or her eyes fixed on a blank screen. During the retrieval

phase, the subject had 4 seconds to decide whether the pattern seen during encoding was on the right or left side of the screen. In the retrieval phase, patterns that differed by as little as a single box were shown, and the subject had a 4-second window to respond. A lack of response was logged as an incorrect response, and feedback appeared at the termination of the 4-second retrieval phase. The response was logged, and the subject was provided real-time feedback on whether the response was correct, was incorrect, or no response was detected. Then, the screen went blank for 11.5 seconds to allow for return hemodynamic response function to return to baseline during the resting phase. . The subjects were instructed to keep eyes fixed on the displayed screen at all times. Each block lasted 26 seconds, and the session consisted of 22 random blocks.

Before administration of methylene blue, our functional MR imaging findings were consistent with previous reports that used the delayed match-to-sample task (8,9). Methylene blue intake was associated with better behavioral performance of the delayed match-to-sample task, and it potentiated functional MR imaging responses during the encoding, maintenance, and retrieval phases of the delayed match-to-sample task.

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