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DAILY CONSUMPTION OF METHYLENE BLUE REDUCES ATTENTIONAL DEFICITS AND DOPAMINE REDUCTION IN A 6-OHDA MODEL OF PARKINSON'S DISEASE

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Abstract—Recently, alternative drug therapies for Parkinson's disease (PD) have been investigated as there are many shortcomings of traditional dopamine-based therapies including difficulties in treating cognitive and attentional dysfunction. A promising therapeutic avenue is to target mitochondrial dysfunction and oxidative stress in PD. One option might be the use of methylene blue (MB), an antioxidant and metabolic enhancer. MB has been shown to improve cognitive function in both intact rodents and rodent disease models. Therefore, we investigated whether MB might treat attentional deficits in a rat model of PD induced by 6-hydroxydopamine (6-OHDA). MB also has neuroprotective capabilities against neurotoxic insult, so we also assessed the ability of MB to provide neuroprotection in our PD model. The results show that MB could preserve some dopamine neurons in the substantia nigra par compacta when 6-OHDA was infused into the medial forebrain bundle. This neuroprotection did not yield a significant behavioral improvement when motor functions were measured. However, MB significantly improved attentional performance in the five-choice task designed to measure selective and sustained attention. In conclusion, MB might be useful in improving some attentional function and preserving dopaminergic cells in this model. Future work should continue to study and optimize the abilities of MB for the treatment of PD.

Key words: 6-OHDA, disengagement behavior, selective and sustained attention, methylene blue, neuroprotection.

INTRODUCTION

Currently, Parkinson's disease (PD) is most commonly treated pharmaceutically with levodopa (L-dopa), which is effective in alleviating many motor symptoms, however L-dopa is often ineffective in restoring certain cognitive functions compromised in PD (Dujardin et al., 1999; Cools et al., 2003; Lewis et al., 2005; Schneider et al., 2013; Robbins and Cools, 2014). Impairments in attentional processes including attention shifting, selective attention, and sustained attention show varied responses to L-dopa in patients with PD. For example, the reduced ability to shift attention to a new rule or task in PD patients is improved with L-dopa (Cools et al., 2002, 2003). However among patients with mild PD, L-dopa has no impact on selective and sustained attention (Lewis et al., 2005; Moustafa et al., 2008). Furthermore, chronic L-dopa administration in patients and in animal models of PD can result in the development of L-dopa-induced dyskinesia (LID) and impulse control disorders (Rajput et al., 2002; Weintraub, 2008; Leeman and Potenza, 2011; Poletti and Bonuccelli, 2013). In a recent study with a rat model of PD (Smith et al., 2016), we also showed that short-term L-dopa treatment was able to restore motor deficits as well as deficits in attentional shifting but prolonged treatment resulted in LID. In the same study L-dopa treatment did not improve performance deficits in a five-choice task that measures selective and sustained attention. For these reasons it is pertinent to investigate alternative treatments for PD.

Here we investigated the possibility of using methylene blue (MB) to treat behavioral and neuronal deficits in a rat model of PD. MB is an antioxidant compound that also increases cell metabolism through the enhancement of mitochondrial activity at the cytochrome oxidase complex (Lindahl and Öberg, 1961; Scott and Hunter, 1966; Visarius et al., 1997). MB has been shown to enhance cognitive function in both intact and disease-modeled rodents. A low dose of MB can facilitate learning and memory of intact rats in both appetitive and aversive contexts by increasing mitochondrial respiration (Callaway et al., 2002, 2004; Martinez et al., 2013). Additionally, chronic MB administration enhanced spatial learning in a mouse model of Alzheimer's disease (Medina et al., 2011) and discrimination learning in a rat model of cerebral hypoperfusion (Auchter et al., 2014). MB was shown to restore motor function and preserve striatal cellular function in a rotenone model of PD

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Abbreviations: LID, L-dopa-induced dyskinesia; MB, methylene blue; NHS, normal horse serum; PB, phosphate buffer; PBS, phosphate-buffered saline; PD, Parkinson's disease; PFA, paraformaldehyde; SNc, substantia nigra; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

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(Wen et al., 2011), but MB's effects on cognitive functions in PD models are unknown.

Mitochondrial dysfunction is a common property of neurodegeneration in PD patients as well as animal models of PD (Janetzky et al., 1994; Mizuno et al., 1998; Fukae et al., 2007; Subramaniam and Chesselet, 2011; Subramaniam et al., 2014), and oxidative stress is considered the primary cause of dopaminergic apoptosis in PD (Kanthsamy et al., 1994; Pallanck and Greenamyre, 2006; Schapira, 2008). Therefore, MB has the potential to be an effective neuroprotective agent by enhancing cell metabolism and reducing reactive oxidative species (Poteet et al., 2012). As a proof of concept, infusion of MB into the striatum directly after an infusion of rotenone to the same site significantly attenuated cell loss at the lesion site (Rojas et al., 2009).

However, as of yet, the ability of MB to restore cognitive and motor deficits and/or simultaneously provide neuroprotection in an animal model of PD has not been shown in the same experimental preparation. Therefore we examined the behavioral and neuronal effects of MB in a unilateral rat model of PD. A five-choice task was used to assess selective and sustained attention. In addition, attentional disengagement/shifting and motor functions (cylinder and pasta tests) were examined. The effects of MB on dopamine cell loss were measured in the same rats tested for attentional and motor functions.

EXPERIMENTAL PROCEDURE

Subjects

Sixty-one Sprague–Dawley male rats (350–450 g) were housed in a reversed light cycle (lights off at 10 AM for 10 h). The rats were food restricted to 90% of free-feeding weight for the duration of the five-choice task training. Water access was restricted for 24 h prior to disengagement testing only. All behavioral training and testing occurred during the dark phase of the light cycle. The rats were divided into four groups with a 2 (dopamine or sham lesion) $\times$ 2 (MB or vehicle feeding) design. All experiments were conducted according to the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. All protocols were approved by the Institutional Animal Care and Use Committee at The University of Texas at Austin. Once immunohistochemical analyses were conducted, twelve dopamine-lesioned rats were deemed to have insufficient lesions and were excluded from further analyses (see Results for detailed explanation).

Surgery

All rats underwent surgery to induce unilateral dopaminergic depletion or sham surgery. First, the rats were anesthetized using 2–5% isoflurane gas (Abbott Laboratories) and were placed into a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). An injection needle was lowered to target the medial forebrain bundle (AP = −3.3, ML = + or −1.7, DV = −8.6). A total of 0.6 μl solution was delivered at 0.1 μl/min via 2-μl Hamilton syringe connected to Harvard apparatus infusion pump. The lesion group received solution containing ~4.2 μg 6-hydroxydopamine (6-OHDA; Sigma–Aldrich, Minneapolis, MN) in 0.1 M phosphate-buffered saline (PBS) with 0.1% ascorbic acid and the sham lesion group received just PBS with 0.1% ascorbic acid). The amount of 6-OHDA injected was chosen with the intent of creating a less-than-severe lesion to better model mild-to-moderate PD in which most attentional dysfunctions are reported (Hornykiewicz, 1974; Filoteo et al., 1997; Zhou et al., 2012). Prior to surgery, all rats were also injected with desipramine (25 mg/kg; Sigma–Aldrich, Milwaukee, WI) to protect noradrenergic cells from 6-OHDA. At the completion of surgery, all rats received a subcutaneous injection of buprenorphine hydrochloride (0.01 mg/kg; TW Medical, Denver, CO, USA) and were placed on a heating pad until they were fully awake.

Daily MB feedings

MB (4 mg/kg, Sigma–Aldrich, Minneapolis, MN) was given orally between 10 and 11 AM daily. MB was dissolved in 10% sucrose water and mixed with 2.6 g crushed Nilla® wafers to create a paste. Blue food coloring (Food, Drug, and Cosmetics Blue No.1) was added to the vehicle solution (10% sucrose water) to form a similar blue paste for the control group.

Behavioral tests

Disengagement test. This test measures the ability to discontinue an ongoing behavior in order to attend to perioral stimulation. Access to water was restricted for twenty-four hours prior to testing. In order to ensure that no somatosensory deficits existed, the rats were tested for basic orienting behavior by stimulating on both sides of the face with a cotton swab out of the rats' sight. During disengagement testing, rats were allowed to drink from a water spigot through a hole in the back wall of the home cage. As rats drank, an experimenter stimulated one side of the face and whiskers using a cotton swab out of the rats' sight. If the rat stopped drinking to attend to the stimulation, this was recorded as a successful disengagement. If the rat continued to drink and did not stop to attend to the stimulation, it was recorded as an unsuccessful disengagement trial. Both sides were stimulated a total of 5 times in varying order.

Cylinder test. This test examines the use of the forelimbs as rats naturally explore the cylinder wall with their forepaws. Rats were placed into a clear Plexiglas cylinder stood on its side (20 cm in diameter and 30 cm in height). During the exploration of the cylinder, spontaneous forepaw touches to the cylinder wall were recorded in accordance with the protocol described in Schallert et al. (2000). If only one paw was placed on the cylinder, it was recorded as independent use of that limb. If both paws were placed on the cylinder simultaneously, or if the other paw was placed immediately after the first paw was placed on the wall, it

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