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Manganese toxicity is targeting an early step in the dopamine signal transduction pathway that controls lateral cilia activity in the bivalve mollusc *Crassostrea virginica*

Michael Nelson, Trevon Adams, Christiana Ojo, Margaret A. Carroll, Edward J. Catapane*

Department of Biology, Medgar Evers College, 1638 Bedford Ave, Brooklyn, NY 11225, USA

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ABSTRACT

Manganese is a neurotoxin causing manganism, a Parkinson-like clinical disorder. Manganese has been shown to interfere with dopaminergic neurotransmission, but the neurotoxic mechanism involved is not fully resolved. In the bivalve mollusc *Crassostrea virginica* also known as the eastern oyster, beating rates of lateral cilia of the gill are controlled by dopaminergic-serotonergic innervation originating from their cerebral and visceral ganglia. Terminal release of dopamine activates D2-like receptors on these gill cells inhibiting adenylyl cyclase and slowing cilia beating rates. In *C. virginica*, manganese treatment disrupts this dopaminergic innervation of the gill, preventing the normal cilio-inhibitory response of lateral cells to dopamine. In this study an adenylyl cyclase activator (forskolin) and two different inhibitors (MDL-12,330A and SQ 22,536) were used to determine if manganese had any effects on the adenylyl cyclase step of the dopamine D2 receptor signal transduction pathway. The results showed that neither the adenylyl cyclase activator nor the inhibitors were affected by manganese in the control of lateral ciliary activity. This suggests that in *C. virginica* the mechanism of manganese toxicity on the dopaminergic control of lateral ciliary activity is targeting an early step in the D2R signal transduction pathway, which may involve interference with D2 receptor activation or alternatively some other downstream signaling activity that does not affect adenylyl cyclase.

1. Introduction

Manganese is a trace element required as an enzyme cofactor or activator for numerous reactions of metabolism (Cotzias, 1958). Excessive manganese exposure in humans can result in neurotoxicity with extrapyramidal symptoms similar to Parkinson's disease (Barbeau, 1984; Calne et al., 1994; Dobson et al., 2004). The primary cause of manganese toxicity is believed due to excessive environmental inhalation (Andersen et al., 1999). The condition was first described in 1837 in two manganese ore-crushing mill workers (Couper, 1837) and has since been referred to as manganism (Mena et al., 1967; Barbeau, 1984; Donaldson, 1987; Gorell et al., 1999). Toxic manganese exposure is also possible in other occupational settings including welding, steel or dry battery manufacturing, and agricultural use of MANEB (manganese ethylene (bis)dithiocarbamate) or other organomanganese fungicides (Olanow, 2004; NAS, 1973; Meco et al., 1994; Reidy et al., 1992; Iregren, 1999). In addition, there is a growing concern about the potential health consequences of chronic low-level ambient manganese exposure in the general population due to increased industrial use of manganese containing compounds being released into the environment as well as commercial use of compounds like methylcyclopentadienyl manganese tricarbony (MMT), an antiknock gasoline additive (Kaiser, 2003).

Human and animal studies have shown toxic manganese exposure results in metal accumulations in various areas of the basal ganglia and dysfunction of cells of both the striatum and the globus pallidus (Calne et al., 1994; Eriksson et al., 1987; Erikson et al., 2004a, b; Brenneman et al., 1999; Nagatomo et al., 1999; Newland, 1999; Pal et al., 1999; Baek et al., 2003). Although manganism has been recognized for some time, the primary mechanism underlying manganese neurotoxicity remains elusive. Considering the clinical similarities between manganism and Parkinson's disease, a dopaminergic cell disorder of the substantia nigra pars compacta, and the fact that manganese accumulates in brain regions rich in dopaminergic neurons, it has long been suggested that manganism involves a disruption in dopaminergic neurotransmission (Neff et al., 1969; Hornykiewicz, 1972; Graham, 1984). Still, the exact mechanism by which manganese produces dopaminergic dysfunction is not fully resolved. While some studies show that manganese selectively

* Corresponding author.
E-mail address: catapane@mec.cuny.edu (E.J. Catapane).

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targets dopaminergic neurons in the human basal ganglia (Olanow, 2004; Pal et al., 1999) and decreases dopamine levels in the striatum (Rosenstock et al., 1971; Eriksson et al., 1987; Parenti et al., 1986; Vescovi et al., 1991; Sistrunk et al., 2007), other reports postulate manganese toxicity is more related to downstream neuronal pathways rather than deficits in nigrostriatal function (Calne et al., 1994; Pal et al., 1999; Huang et al., 1998; Olanow, 2004).

In many bivalve molluscs, including *Mytilus edulis* and *C. virginica*, beating rates of lateral cilia are controlled *via* the branchial nerve by a reciprocal dopaminergic and serotonergic innervation originating from their cerebral and visceral ganglia (Bayne, 2017). Dopamine is cilio-inhibitory and serotonin is cilio-excitatory (Catapane et al., 1978; Aiello, 1990; Carroll and Catapane, 2007). Application of dopamine directly to the ganglia or stimulating the branchial nerve (20 Hz, 2 ms duration, 10 V) results in a terminal release of dopamine at the gill, decreasing the beating rates of the lateral cell cilia (Catapane et al., 1978; Martin et al., 2008; Nelson et al., 2010). In *C. virginica*, direct application of dopamine to isolated gill reduces lateral ciliary activity in a dose dependent fashion (10^{-7}–10^{-3} M) with 10^{-5} M as the ED50 dose (Carroll and Catapane, 2007).

As filter feeders, the bivalve molluscs accumulate environmental toxins including heavy metals (Cunningham, 1979). The tissues of *C. virginica* were found to readily accumulate various heavy metals, including manganese (Rodney et al., 2007; Murray et al., 2007). When *C. virginica* were treated for 3 days with two different doses of manganese, the lateral ciliary response to direct application of dopamine (10^{-7}–10^{-3} M) was significantly impaired, while having no effect on the cilio-excitatory response to applied serotonin (Martin et al., 2008). While low dose manganese treatment (50 μM) caused partial impairment of the cilio-inhibitory response to dopamine, high dose manganese treatment (500 μM) completely blocked the dopamine dose response. In those experiments, the fact that there was impairment of the cilio-inhibitory response to direct application of dopamine to gills of manganese treated oysters strongly suggested that at least one aspect of manganese toxicity is due to a post-synaptic issue rather than a reduction of dopamine release at the presynaptic terminal.

All dopamine receptors belong to a large superfamily of G protein coupled metabotropic receptors (GPCR), whose actions are mediated by a subset of the 16 heterotrimeric G protein subtypes, functionally classified into four broad classes which include $G_{\alpha s}$ that activates adenylyl cyclase and $G_{\alpha i}$ that inhibits adenylyl cyclase (Pierce et al., 2002). Immunohistofluorescence studies demonstrated the dopamine receptors in lateral cells of the gill in *C. virginica* are D2-like (Anador et al., 2011). In other experiments, Catapane et al. (2016) showed that the cilio-inhibitory effects of dopamine application to the gill or branchial nerve stimulation (20 Hz) resulted in lateral cell membrane hyperpolarization, which is in agreement with D2-like receptor activation as reported in vertebrates and various invertebrates (Momiyama et al., 1993; Zhong et al., 2013).

The D2-class dopamine receptors are coupled to the $G_{\alpha i/o}$ family of G proteins, which inhibits adenylyl cyclase lowering levels of cAMP by (Neves et al., 2002; Rankin et al., 2010; Beaulieu and Gainetdinov, 2011). Since the cell signaling pathway of activated D2 receptors (D2R) involves adenylyl cyclase inhibition, this study examined the effects of directly activating and inhibiting adenylyl cyclase on lateral cilia activity in manganese treated gill of directly activating and inhibiting adenylyl cyclase in order to physiologically determine whether manganese toxicity disrupted dopamine signaling before or after the adenylyl cyclase step in the D2R signal transduction pathway.

2. Materials and methods

2.1. Animals

Adult *C. virginica* of approximately 80 mm shell length were obtained from Blue Island Oyster Company, Sayville, NY, USA. They were maintained in the lab for up to two weeks in temperature-regulated aquaria in Instant Ocean® artificial seawater (ASW) obtained from Aquarium Systems Inc. (Mentor, OH, USA) at 16–18 °C, specific gravity of 1.024 ± 0.001, salinity of 31.9 ± 1.3 and pH of 7.8 ± 0.2. Prior to experimentation each animal was assessed for the degree of resistance it offered to being opened. Animals that fully closed in response to tactile stimulation and required at least moderate hand pressure to being opened were considered acceptable for use in the experiments.

2.2. Chemicals

Dopamine, the adenylyl cyclase activator forskolin, and the adenylyl cyclase inhibitors MDL-12,330A (cis-*N*-(2-Phenylcyclopentyl)-azacyclotridec-1-en-2-amine hydrochloride) and SQ22,536 (9-(Tetrahydro-2-furanyl)-9H-purin-6-amine) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other reagents including manganese chloride ($MnCl_2{\cdot}4H_2O$, ASC grade) were obtained from Fisher Scientific (Pittsburgh, PA, USA). A stock solution of $MnCl_2$ (0.1 M) was made in dH_20 and diluted to 100 μM in ASW. Stock solutions of forskolin, MDL-12,330A and SQ22,536 were prepared in DMSO (10^{-2} M) and diluted to working solutions with ASW. Just prior to use a stock dopamine solution was prepared in dH_20 and then diluted to working solutions with ASW containing 10 mg% ascorbic acid (ASWA) buffered with sodium bicarbonate, pH 7.8, to retard dopamine oxidation as described by Malanga (1975).

2.3. Determination of lateral cilia beating rates

Gills were dissected from animals and sections of approximately 2.5 cm were positioned on a microscope slide with a cover slip. The cover slip was supported by thin plastic spacers to prevent crushing of the filaments and allow for fluid movement between the slide and cover slip. Gill filaments were viewed at 200× magnification with a microscope fitted with transmitted stroboscopic light from a Grass PS 33 Plus Photic Stimulator. Beating rates of lateral cilia were measured by the method of Catapane et al. (1978) by synchronizing the flashing rate of the stroboscope with the beating of the cilia. Because the lateral cilia beat in a metachronal wave pattern (Aiello and Sleigh, 1972), synchronization is achieved when the lateral cilial waves appear motionless in a characteristic horse-shoe like configuration. At all multiple synchronizing rates above the one corresponding to the true beating frequency, the wavelength of the beating cilia will appear to be a fraction of the true wavelength. Cilia beating rates are expressed as beats/s ± sem. Lateral cells of isolated gills tend to have either quiescent cilia or cilia that are actively beating at about 10–15 beats/s. Only excised gill preparations with actively beating cilia were selected to test for cilio-inhibitory effects in order to eliminate the necessity of first artificially activating the cilia with serotonin.

2.4. Statistics

Data were analyzed using the Student's *t*-test.

3. Results

3.1. Effects of manganese on the dopaminergic control of lateral ciliary activity

A dose response to dopamine (10^{-7}–10^{-3} M) was conducted on manganese treated excised gills to determine effects on beating rates of lateral cilia compared to controls. Dopamine doses were administered and cilia rates determined after 10 min intervals. Fig. 1 shows the expected progressively decreasing cilia beating rates in control gills with increasing dopamine concentrations.

The ED50 for dopamine was 10^{-5} M as previously reported by Carroll and Catapane (2007). In gills acutely treated with manganese