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Novel NMDA receptor-specific desensitization/inactivation produced by ingestion of the neurotoxins, β-N-methylamino-L-alanine (BMAA) or β-N-oxalylamino-L-alanine (BOAA/β-ODAP)

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ABSTRACT

The environmental neurotoxins BMAA (β-N-methylamino-L-alanine) and BOAA (β-N-oxalylamino-L-alanine) are implicated as possible causative agents for the neurodegenerative diseases, amyotrophic lateral sclerosis/ParkinsonismDementia complex (ALS/PDC) and neurolathyrism, respectively. Both are structural analogs of the neurotransmitter, glutamate, and bind postsynaptic glutamate receptors. In this study, the effect of ingestion of these toxins on the response of a singly-innervated, identified, glutamatergic postsynaptic cell in a living, undissected *Drosophila* is observed by intracellular recording. Previously we have reported that ingested BMAA behaves as an NMDA agonist that produces an abnormal NMDA response in the postsynaptic cell. It is shown here that BOAA also behaves as an NMDA agonist, and produces an effect very similar to that of BMAA on the postsynaptic response. In response to a single stimulus, the amplitude of the NMDA component is decreased, while the time to peak and duration of the NMDA component are greatly increased. No discernable effect on the AMPA component of the response was observed. Furthermore, both BMAA and BOAA cause an NMDAR-specific desensitization in response to repetitive stimulation at the physiological frequency for the postsynaptic cell (5 Hz). The possibility that this phenomenon may represent a response to excessive Ca^{2+} entry through NMDAR channels is discussed. This desensitization phenomenon, as well as the abnormal NMDAR gating characteristics induced by BMAA, appears to be rescued during higher frequency stimulation (*e.g.* 10, 20 Hz).

1. Introduction

Evidence suggests that human consumption of foodstuffs containing the neurotoxins, BOAA (β-N-oxalylamino-L-alanine) or BMAA (β-N-methylamino-L-alanine), is linked to the onset of neurodegenerative disease. BOAA (also known as β-ODAP [β-N-oxalyl-L-α,β-diaminopropionic acid]) is found in the grass pea (*Lathyrus sativus*), a drought resistant legume grown in Asia and East Africa. Excessive consumption of the grass pea in times of famine is associated with development of the neurodegenerative disease, neurolathyrism (Rao et al., 1964; Spencer et al., 1986), which is characterized by degeneration of pyramidal tract neurons in the spinal cord (Striefler et al., 1977; Ravindranath, 2002) and leg controlling neurons in the cortex (Haimanot et al., 1990), resulting in irreversible leg paralysis.

BMAA, on the other hand, is produced by a ubiquitous cyanobacteria (Adams, 2002; Cox et al., 2005), and is found in food supplies all over the world. It was first associated with the elevated incidence of amyotrophic lateral sclerosis/Parkinsonism dementia complex (ALS/PDC) on the island of Guam (Whiting, 1988), where the diet of the indigenous population included flour made from the BMAA-containing cycad plant, as well as animals that fed on the plant. Many *in vivo* animal studies have been performed that demonstrate the neurotoxicity of ingested BMAA (for review, see Chiu et al., 2011). However, the causative effect of BMAA in ALS/PDC was questioned because it was felt that many of these studies used high concentrations of BMAA that could not be achieved by human consumption (Duncan et al., 1990). However, it has since been demonstrated that BMAA is biomagnified in the food chain (Banack and Cox, 2003; Brand et al., 2010; Jonasson et al., 2010; Mondo et al., 2012), so that it can be consumed in sufficient quantities to be toxic. Furthermore, elevated levels of BMAA have been found in the postmortem brain tissue of ALS/PDC patients in Guam and patients with Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS) in North America, but not in control brains (Murch et al., 2004; Pablo et al., 2009), suggesting a possible role in the etiology of these diseases. Thus, long term consumption of plants containing BMAA (or animals that have biomagnified it) may be one risk factor in the delayed onset of the neurodegenerative diseases, ALS, AD or PD.

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Both BOAA and BMAA [in its carbamylated form (Weiss and Choi, 1988)] are glutamate analogs. L-BOAA has been reported to be an agonist of glutamate receptors, particularly α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) (Bridges et al., 1989; Ross et al., 1989). Evidence suggests that BMAA is an agonist of various glutamate receptors, including AMPA/kainate receptors (Rakonczay et al., 1991; Rao et al., 2006) and NMDA (N-methyl-D-aspartate) receptors (Lindstrom et al., 1990; Copani et al., 1991).

With both of these molecules, excitotoxicity, the cascade of toxic events initiated by excessive intracellular Ca^{2+}, appears to be a likely mode of action. While the mechanism of neurotoxicity of the BOAA molecule is not completely understood, various lines of evidence suggesting that excitotoxicity initiated by overstimulation of glutamate receptors (for review, see Van Moorhem et al., 2011) or elevation of extracellular glutamate by interference with glutamate transport systems (Ross et al., 1985; Warren et al., 2004) have been presented. Toxicity by Ca^{2+} accumulation in the postsynaptic cell has been reported (Kusama-Eguchi et al., 2014). With BMAA, increases in the intracellular Ca^{2+} concentration resulting from glutamate receptor activation have been demonstrated in various preparations (Brownson et al., 2002; Rao et al., 2006; Cucchiaroni et al., 2010).

This paper investigates by intracellular recording the effect that ingesting BOAA or BMAA has on the response of a singly-innervated, identified glutamatergic postsynaptic cell in a living, undissected organism (*Drosophila*). The response is elicited by stimulation (either singly or repetitively) of the motor neuron through the giant axon pathway. Both stimulating and recording electrodes are inserted directly through the cuticle, causing essentially no damage to the organism. After the experiment, the fly can be returned to its vial for follow-up experiments. This preparation is extremely well-suited for observing the effect of ingested substances on a specific glutamatergic postsynaptic cell. For example, it is possible to observe the progressive deterioration of the cell response over time by recording from the identical cell of the identical fly multiple times over the course of a week's feeding. Furthermore, recovery of a particular cell can be observed by switching from toxin-containing to non-toxin-containing medium. Another advantage of this preparation is that the AMPA and NMDA components of the postsynaptic response are identifiable in the response waveform, allowing determination of which receptor type is impacted, as well as how it is impacted.

We demonstrate here that in response to a single stimulus, BOAA behaves as an NMDA agonist causing abnormal opening and closing times of the NMDARs, which results in a greatly prolonged NMDA response. This effect is very similar to that observed for BMAA, already reported (Goto et al., 2012).

Furthermore, we report that with repetitive stimulation at 5 Hz, which is the frequency experienced by this cell during normal physiological activity (flight), both BOAA and BMAA demonstrate an unusual, NMDA-specific desensitization/inactivation, which causes the amplitude of the NMDA component of the postsynaptic response to quickly decline, and the duration to shorten (for the remainder of this paper, this phenomenon will be referred to as desensitization, since it occurs in the continuous presence of the NMDA agonists). The AMPA response, on the other hand, remains unaltered during this stimulation regimen. In BMAA-fed flies, this desensitization phenomenon appears to be rescued by a higher frequency stimulation (10, 20 Hz), and the response becomes more normal in appearance, increasing in amplitude and decreasing in duration as long as the stimulation continues. The possibility that the NMDA-specific desensitization may represent a response of the postsynaptic cell to an abnormally large increase in the intracellular [Ca^{2+}] is discussed.

2. Materials and methods

Three-day-old virgin female *Drosophila melanogaster* of the wild-type strain, Oregon-R, were used in this study. The flies were raised on a standard medium composed of cornmeal, yeast, agar, sucrose, dextrose and trace minerals. On the third day, the flies were transferred to vials containing 750 μL standard medium to which was added 250 μL of 100 mM solution of BMAA or BOAA. Control flies were transferred to vials containing 1 mL of the standard medium.

2.1. Physiology

Intracellular recordings were made from the fibers of the dorsal longitudinal flight muscle (DLM), which powers the downward stroke of the wing during flight. A bilateral pair of DLM muscles, each composed of 6 fibers, runs the length of the thorax, attaching anteriorly to the dorsal thoracic cuticle and posteriorly to the posterior phragma. Muscle fibers 1–4 are each singly innervated by four glutamatergic motor neurons (MN1-4) (Ikeda et al., 1980) that are located in the thoracic ganglion (Ikeda and Koenig, 1988). For these experiments, ipsilaterally located fibers 2 and 3 were used. A more complete description of the recording technique can be found in previous publications (Koenig and Ikeda, 2005; Goto et al., 2012). Briefly, the fly was immobilized in Tackiwax with the dorsal thorax exposed, and the abdomen free to move, allowing normal respiration. The recording electrode (glass micropipette) was inserted directly through the cuticle into the muscle fiber at its anterior attachment point. The ground electrode (fine steel pin) was inserted shallowly into the scutoscutellar suture at the midline. The two stimulating electrodes (fine steel pins) were inserted through the cuticle into the brain of the fly on either side of the postfrons in between the orbital setae to stimulate the giant interneuron. A 0.1 ms duration square pulse was applied for stimulation of the DLM motor neurons. Test pulses below the threshold for the giant interneuron were applied, until threshold for firing was obtained. This threshold is approximately 4–6 μA (depending on electrode insertion locations), which is well below firing for any other neuron in the brain, the giant interneuron being so much larger. The electrical responses of the muscle fibers were fed into a MacLab/4 SP data acquisition system and stored in a Macintosh G4 computer.

After the recording, the electrodes were removed and the fly was released from the Tackiwax and returned to its vial with toxin-containing medium. In some cases, the fly was returned to a vial with non-toxin-containing medium. These flies were recorded again on subsequent days to observed successive days of feeding on toxin-containing medium or recovery.

In one experiment, a small piece of cuticle was removed at the humerus, and a 0.1 μL droplet of 200 μM MK80l in saline was applied over the small opening using a small glass micropipette attached to a tube. The droplet was applied by gently blowing into the tube. The droplet is quickly absorbed into the body of the fly, and the haemolymph soon coagulates, closing the opening. This was done with the stimulating, recording, and ground electrodes in place. The fibers were then stimulated to observe the effect of MK801 on the postsynaptic response.

2.2. Calculations

In order to demonstrate the differences in response waveform between toxin-fed and non-toxin-fed flies, the following averages were calculated: (1) time to peak, measured from the onset of the AMPA response (the AMPA response depolarizes in less than a ms to threshold point for NMDA response) to the point of maximum amplitude; (2) amplitude, measured from the resting level of the cell to the greatest point of depolarization; and (3) duration, measured from the onset of the AMPA response to the point where the response returns to resting level. Standard deviations (±) were calculated for averages.

2.3. Behavior

BOAA-fed flies were tested for their ability to climb, fly, walk, and stand (BMAA-fed flies have been tested previously [Goto et al., 2012]).