

Conotoxins: Possible Therapeutic Measure for Huntingtons Disease

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Abstract

Huntington's disease (HD) is a genetic disorder with autosomal dominant inheritance with progressive degeneration of neurons. It is characterized by affective, cognitive, behavioral, and motor dysfunctions. 3 nitropropionic acid is well established animal model which initiates a complex series of neurochemical and signaling changes that lead to pathological events including neuronal excitotoxicity by excessive glutamate release, neuroinflammation and energy impairment. Excitotoxins like NMDA and kainic acid which binds to these receptors to release high levels of glutamate that can cause excitotoxicity by allowing high levels of calcium ions (Ca^{2+}) to enter the cell. Ca^{2+} influx into cells activates a number of enzymes, including phospholipases, endonucleases, and proteases such as calpain. These enzymes increase apoptosis, oxidative stress and energy impairment thereby causing mitochondrial dysfunctions and go on to damage neuronal structures. The toxic venom used by the cone shells called conotoxin contains up to 50 different peptides that selectively inhibit the function of ion channels and excitatory amino acid receptors such as NMDA involved in the transmission of nerve signals in animals representing an extensive array of ion channel blockers each showing a high degree of selectivity for particular types of channels. Conotoxins selectively target a range of ion channels and NMDA receptors, making them widely useful tool for probing nervous system function. Here we hypothesize the protective effect of conotoxins, from genus *Conus geographus* named w-conotoxin and conantoxin, against excitotoxic neuronal cell death using 3 NP induced Huntington's model.

Keywords: Huntington's disease (HD); Neurodegeneration; Excitotoxicity; Conopeptides; Conotoxins; NMDA; 3 Nitropropionic acid

Introduction

Huntington's disease (HD) is a genetic disorder with autosomal dominant inheritance with progressive degeneration of neurons. It is characterized by affective, cognitive, behavioral, and motor dysfunctions preferentially due to morphological abnormalities and cell death of the striate medium-sized spiny neurons. 3-Nitropropionic acid (3-NP), a neurotoxin irreversibly inhibits succinate dehydrogenase enzyme (complex-II) in the electron transport chain and produces HD like symptoms both in animals and human [1]. 3-NP intoxication leads to selective striate lesions which begin in the striatum and later spread to the entire brain [2]. Studies reported that 3-NP treatment significantly causes oxidative damage in diverse areas of brain particularly striatum. Possible source of oxidative damage in 3-NP model is impairment of mitochondrial energy production (inhibition of complex-II) [3,4]. Excitotoxicity contributes to the pathogenesis of HD [5,6]. Studies in both rodents and primates show striking similarities between striatal lesions produced by N-Methyl-D-Aspartate (NMDA) agonists and the neurochemical and histologic features of HD [7-10], that causes an increase in oxygen flux through mitochondria, leading to elevated ROS production [5,11].

Conus species i.e. *conus geographus* contains a unique set of peptides in their venom specifically ω (omega)-conotoxin and conantokins. These predatory marine snails (cone shells) inject this potent cocktail of peptide toxins into their prey to immobilise them [12,13]. The toxic venom used by the cone shells contains up to 50 different peptides that selectively inhibit the function of voltage gated N type of Ca^{++} ion channels involved in the transmission of nerve signals in animals and antagonizes the function of NMDA receptors [14,15]. So these toxins that are used for defence by animal could be isolated, tested and used for various pharmacological and physiological activities. This hypothesis focuses the properties of conopeptides [16] and to use them as drug leads for the development of novel therapeutics for the treatment of Huntington's disease (HD) occurring due to glutamate toxicity.

Conotoxins

Venomous animals have evolved a vast array of peptide toxins for prey capture and defence. These predatory animals have devised their efficient venom apparatus that allows them to successfully capture polychaete worms, molluscs or in some cases fish as their primary food sources [12]. Marine cone snails from the genus *Conus* are estimated to consist of up to 700 species. They have been previously grouped into superfamilies according to signal sequence and into families based on their cysteine framework and biological target [13]. The number of conotoxins whose activities have been determined and they are called the α (alpha)-, δ (delta)-, κ (kappa)-, μ (mu)-, conantokins and ω (omega)-types. The high specificity exhibited by these novel compounds for neuronal receptors and ion channels in the brain and nervous system indicates the high degree of selectivity that this class of neuropeptides can be expected to show when used therapeutically in humans.

Each of the types of conotoxins attacks a different ion channel. α -conotoxin inhibits nicotinic acetylcholine receptors at nerves and muscles. δ -conotoxin inhibits the inactivation of voltage-dependent sodium channels. κ -conotoxin inhibits potassium channels. μ -conotoxin inhibits voltage-dependent sodium channels in muscles. ω -conotoxin inhibits N-type voltage-dependent calcium channels [12-14]. N-type voltage-dependent calcium channels are located in the pain and excitatory amino acid receptors of the nervous system, ω -conotoxin are inhibitors of voltage gated Ca^{++} channels and also

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has an analgesic effect, whereas conantokins are antagonists of NMDA (an excitatory amino acid) receptor [4,14]. These ω -conotoxin and conantokins are reported to reduce glutamate toxicity [4,17].

Purification of conopeptides

Conotoxins are multiple disulfide-bonded peptides isolated from marine cone snail venom. These toxins have been classified into several families based on their disulfide pattern and biological properties. The venom of cone snails can be extracted from their venom glands. The peptides from conotoxins from conotoxins can be separated and eluted by reverse phase HPLC. The molecular masses of the peptide scan are determined by electro spray ionization mass spectrometry. Further purification and synthesis of peptides can be done by solid phase peptide synthesis method and amino acid analysis can be done by amino acid analyzer followed perhaps by NMR-based structure determination [18].

The chemical re-engineering of these via cyclization has been particularly valuable in improving their biopharmaceutical properties. A variety of other chemical re-engineering approaches have also been used. Minor re-engineering of χ -conotoxin Mr1a to convert its N-terminal residue to pyroglutamic acid proved particularly successful and the modified derivative, Xen2174, is currently in clinical trials for neuropathic pain. A lead compound, ACV1 (conotoxin Vc1.1 from *Conus victoriae*), has entered Phase II clinical trials and is being developed for the treatment of neuropathic pain. ACV1 will be targeted initially for the treatment of sciatica, shingles and diabetic neuropathy. The compound is a 16 amino acid peptide a novel alpha-conotoxin identified by gene sequencing, an antagonist of neuronal nicotinic acetylcholine receptors is active in suppressing the vascular response to selective stimulation of sensory nerves in vivo [19].

The rationale for the role of excitotoxicity in the pathogenesis of Huntington's disease

Excitatory synaptic transmission in the mammalian CNS is principally mediated by L-glutamate. Glutamate is the major excitatory transmitter in the brain. Glutamate information which regulates brain development and that determines cellular survival, differentiation and elimination as well as formation and elimination of nerve contacts (synapses) [17,20]. Glutamate interacts with at least three classes of membrane receptors, each commonly referred to by preferred pharmacological agonists: N-methyl-D-aspartate (NMDA), quisqualate, and kainate [21]. These three classes are linked to membrane cation channels. A normal cellular uptake mechanism removes excess glutamate from the extracellular space [22,23]. The toxic changes produced by glutamate or related excitatory amino acids in vivo are of two sorts: possibly corresponding to the acute swelling and delayed degeneration observed in vivo [24]. The first component, marked by acute neuronal swelling, depends on extracellular Na^+ and Cl^- and can be mimicked by other depolarizing agents [25]. The second component, marked by delayed neuronal degeneration, depends on extracellular Ca^{++} and may be thus mediated by the toxic effects produced by excessive Ca^{++} influx [22,24,25]. Glutamate-induced Ca^{2+} accumulation by cortical neurons is closely correlated with resultant neuronal degeneration [26]. There is no proven effective therapy for Huntington's disease exists, It will be important to determine whether certain more effective treatments or have fewer side effects than others in disease states might be good approach to enhance the disease tolerance. NMDA receptor antagonist could be beneficial neuroprotective agents or ion channel blockers which

inhibit glutamate-induced translocation of protein kinase C and thereby reducing excitotoxic neuronal cell death [4,20,27].

Hypothesis

Currently, there is no therapy available which can stop or postpone huntingtons disease. We can only slow down the progression of the disease. Though it is unexplained why the mutation of gene takes place and other mechanisms related to it, the facts like oxidative stress, excitotoxicity and mitochondrial dysfunctions leads to the fatal behavioural, motor and cognitive manifestations of the disease. Although many therapies have been proposed for the treatment, there is no therapy which can entirely cure this disease. Whereas, there are some therapies which give symptomatic relief or minimize the oxidative stress of the body and that almost all of them have considerable shortcomings. There is need to find out some more potent treatment that could reduce the neuronal cell death. Our literature review showed that ω -conotoxin and conantokins acts as the NMDA receptor antagonists as well as reduces the Ca^{++} signaling and extracellular glutamate release and thereby minimizing excitotoxicity, mitochondrial dysfunctions and energy impairments, enhancing the life span of neurons. On the basis of this approach the studies on the animal model of Huntington's disease could be performed. If this hypothesis is shown to be effective, through preclinical studies, it would have important implications for management of Huntington's disease as well as many other neurodegenerative diseases.

Evaluation of hypothesis

Series of neurochemical, neurophysiological and structural abnormalities are observed in the CNS due to HD. The prominent manifestations seen in Huntington's disease are cognition impairment due to neurodegeneration with loss of working memory, choreoforms, loss in coordination and balance, slurred speech and difficulty in writing. All these abnormalities are due to decreased succinate levels and increased lactate levels. Many emotional changes occur during progression of the disease in which depression and irritability are prominent psychiatric symptoms [28]. Evaluation of hypothesis can be done by estimating behavioral and biochemical parameters like muscular grip strength using rotarod apparatus, locomotor activity. Cognition impairment is evaluated by morris water maze. In huntington's disease biochemical enzymes in the brain are majorly hampered. Estimating levels of the enzymes may be useful to check the effectiveness of the treatment. Biochemical parameters like succinate dehydrogenase activity, lactate dehydrogenase activity, Oxidative stress parameters such as MDA, NO, catalase, SOD activity are the important aspects that may be useful in the evaluation of the hypothesis. Detailed description of the methods to evaluate conotoxins against animal model of huntingtons diseases are given below.

Rotarod activity

Motor in coordination and grip strength was assessed by using rotarod apparatus (Techno, Ambala, India). Animals were exposed to prior training session to acclimatize them on rotarod before starts the actual assessing of drug treatment. Animals were placed on the rotating rod with a diameter of 7 cm (speed 25 rpm). The cut off time was 180s. Three separate trials after 5 min gap were given to each rat. The average fall of time was recorded and expressed as count per 5 min [29].

Assessment of gross behavioral activity (locomotor activity)

The locomotor activity was monitored by using actophotometer

(IMCORP, Ambala, India). The motor activity in actophotometer was detected by infrared photo cells installed in the instrument. Animals were placed individually in the activity chamber for 3min as a habituation period before making actual recording. Each animal was observed over a period of 5min and expressed as counts per 5min [30].

Morris water maze test

The acquisition and retention of a spatial navigation task was examined using Morris water maze [5]. Animals were trained to swim in circular pool (180 cm diameter×60 cm height) located in a test room. The pool was filled with water ($28 \pm 2^\circ\text{C}$) to a depth of 40 cm. A movable circular platform 9 cm in diameter, mounted on a column was placed in the pool 1 cm above the water level for maze acquisition test. The platform was fixed in the center of any one of the 4 quadrants and remained in that location for the duration of the experiment. Animals received a training session consisting of 4 trials in each session for 4 days, starting from the first day of 3-NP administration. In all 4 trials, starting positions were different. The latency to find the platform was recorded up to a maximum of 2 min. The time taken (latency) to reach the platform on the 5th day was recorded as initial acquisition latency. On the 10th and 15th day, the platform was removed. Each animal was randomly released from any one of the edges (N, S, E, W) facing the wall of the pool. The time taken to reach the quadrant where platform was placed was recorded.

Measurement of Oxidative Stress Parameters

Measurement of lipid peroxidation

The quantitative measurement of lipid peroxidation in striatum was performed according to the method of Wills [31]. The amount of malondialdehyde (MDA), a measure of lipid peroxidation was measured by reaction with thiobarbituric acid at 532 nm using Perkin Elmer lambda 20 on spectrophotometer (Norwalk, CT, USA). The values were calculated using molar extinction coefficient of chromophore ($1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$) and expressed as percentage of vehicle treated group.

Estimation of nitrite

The accumulation of nitrite in the supernatant, an indicator of the production of nitric oxide (NO), was determined with a colorimetric assay with Greiss reagent (0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide and 2.5% phosphoric acid) as described by Green et al. [32]. Equal volumes of supernatant and Greiss reagent were mixed, and incubated for 10 min at room temperature. The absorbance of each sample was determined at 540 nm at Perkin Elmer lambda 20 spectrophotometer. The concentration of nitrite in the supernatants was determined from a sodium nitrite standard curve and expressed as percentage of vehicle treated group.

Catalase estimation

Catalase activity was assayed by the method of Luck [33], wherein breakdown of hydrogen peroxides (H_2O_2) is measured at 240 nm. Briefly, assay mixture consisted of 3 ml of H_2O_2 phosphate buffer and 0.05 ml of supernatant of tissue homogenate (10%), and change in absorbance of each sample was recorded at 240 nm. The results were expressed as micromole H_2O_2 decomposed per milligram of protein/min.

Superoxide dismutase activity (SOD)

SOD activity was assayed according to the method of cono [34] where in reduction of nitrazolbluetetrazolium (NBT) was inhibited

by the SOD is measured at 560 nm using spectrophotometer. Briefly, reaction was initiated by the addition of the hydroxylamine hydrochloride to the mixture containing NBT and sample. The results were expressed as unit/mg protein.

Succinate dehydrogenase (SDH) activity (Complex-II)

SDH was measured spectrophotometrically according to King [35]. The method involves oxidation of succinate by an artificial electron acceptor, potassium ferricyanide. The reaction mixture contained 0.2 M phosphate buffer pH 7.8, 1% BSA, 0.6 M succinic acid, and 0.03 M potassium ferricyanide. The reaction was initiated by the addition of mitochondrial sample and change in absorbance was recorded at 420 nm.

Lactate dehydrogenase enzyme activity

The Lactate dehydrogenase enzyme activity was estimated by the method of King [35]. The reaction mixture contained 0.5 ml of buffered substrate made in 0.1 M glycine buffer (pH 7.9) and 0.1 ml of tissue homogenate. The tubes were allowed to be incubated for 30 min at 37°C . Finally, the color was intensified by the addition of 5.0 ml of 0.4 N NaOH. The absorbance was read at 440 nm. The activity was expressed as pyruvate formed/min/mg protein.

Statistical analysis

The data was analyzed by using analysis of variance (ANOVA) followed by Tukey's test. All the values are expressed as mean \pm S.E.M. In all tests, the criterion for statistical significance was $P < 0.05$.

Conclusion

Huntingtons disease causes neurodegeneration due to excitotoxicity and mitochondrial dysfunction. The key role of Conotoxins in reducing Ca^{2+} influx and NMDA receptor antagonism thereby reducing glutamate excitotoxicity could be important consideration as therapeutics. So this hypothesis suggests the animal studies of conotoxin against 3 nitropropionic acid induced huntingtons model. Once validated, Conotoxins may serve as a target drugs in HD management.

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Conflict of Interest

There are no any conflicts of interest.

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