



Aequorin

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Neurodegenerative disorders (like Alzheimer's, and Parkinson's) pose a serious health threat to our families and national resources. Alterations in the capacity to maintain normal calcium homeostasis have been suggested to underlie the reduced cellular function characteristic of the aging process, and to predispose the senescent organism to a host of diverse pathologies including cancer, heart disease, and a range of muscle and neurodegenerative diseases (Squier et al., 2000).

The role that CaBPs play in the human body as protective agents have also been widely recognized (Heizmann and Braun, 1992). The increasing cost of caring for the elderly and the advancing age of the U.S. and world population mandates intensive research aimed at understanding aging-related changes in brain regions important for learning and memory, and developing and evaluating potential therapeutics for these disorders.

While a healthy body has a normal calcium balance, persons with neurodegenerative diseases lack such a balance. The scientific literature suggests that a substance that can restore calcium balance would preserve physical health and could treat persons who have already begun to decline. Initial studies will investigate the extent to which aequorin gets into neurons in key brain regions known to be affected by the aging process, and then begin to investigate how administration of aequorin alters neuronal function and whether any observed changes result in improved cognitive function in aged animals.

Quincy Bioscience, LLC (Madison, WI) in collaboration with the University of Wisconsin-Milwaukee is researching such a substance, which is called aequorin. In nature it is found in miniscule quantities in jellyfish. Aequorin is from a family of CaBPs known as the EF-hand family. EF-hand proteins also constitute the other CaBPs endogenous to the human body. Aequorin and many of these endogenous proteins are homologous in structure and show strong nucleotide sequence similarity (Moncrief et al., 1990, Tsuji et al., 1995).

Preliminary research by Quincy has found aequorin holds great promise. The company plans to research and develop novel calcium-binding proteins for the prevention and treatment of neurodegenerative disorders. We are looking for the right marketing and development partners. The calcium-binding ability of aequorin is well known, however no other lab has explored its therapeutic potential. In part this is due to the scarcity of the substance. Two tons of jellyfish are needed to yield 125 mg. of aequorin. Aequorin was unavailable and expensive. Recently developed recombinant production techniques have made Aequorin manufacture and purification easier (Shimomura and Inouye, 1999), and bring with it the possibility of using it as a therapeutic.

The overall goal of this proposal is to develop and evaluate a novel therapy involving administration of the calcium binding protein aequorin for the treatment of aging-related cognitive decline and neurodegenerative disorders. The therapeutic would ideally be administered as in an orally acceptable form, however, inhalable, intravenous or other routes of administration could be utilized. Should aequorin prove useful in treating degenerative disorders, it will open up the door to developing increasingly effective treatments for neurodegenerative disorders based on CaBPs.

Aging-related neuronal disorders pose a serious health threat to our families and national resources. Alzheimer's disease affects ~30% of all people over the age of 85. Nearly 14 million people may suffer from Alzheimer's disease by the year 2040. The increasing cost of caring for

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the elderly and the advancing age of the U.S. and world population mandates intensive research aimed at developing treatment strategies. Over the last fifteen years, a unifying hypothesis called the calcium hypothesis of aging and dementia has emerged. It posits that dysregulation of calcium homeostasis is a primary factor contributing to aging-related learning and memory impairments observed in many species, including humans.

Background:

The need for novel treatments for age-related cognitive decline. The importance of intracellular calcium: Calcium is an essential second messenger that plays important roles in a plethora of neuronal functions, including synaptic plasticity, activation of kinases and phosphatases, regulation of gene expression, and excitotoxic cell death plasticity (Williams and Johnston, 1989; Bröcher et al., 1992; Uchitel et al., 1992; Choi, 1994; Yeckel et al., 1999). The latter is particularly important because, although calcium is vital to normal neuronal function, it is very tightly regulated and too much calcium is actually detrimental. Excessive calcium influx is particularly troublesome for aging neurons because they are less able to handle excessive calcium influx, at least partly due to decreases in calcium binding proteins, (CaBPs) which help to buffer intracellular calcium. A number of studies suggest that when aged neurons are activated, they experience excessive calcium influx – particularly those neurons in brain regions vital for normal memory, such as the hippocampus and associated medial temporal lobe (MTL) structures (Moyer et al., 1992; Moyer and Disterhoft, 1994; Moyer and Brown, 1998; Moyer et al., 2000). With such a pivotal role in neuronal function, it is not surprising that calcium has been intensely studied in the fields of learning, memory, and aging.

Calcium Hypothesis of Aging: Over the last fifteen years, a unifying hypothesis has emerged which attempts to explain some of the cognitive deficits seen in aging. This hypothesis is referred to as the calcium hypothesis of aging (Khachaturian, 1987; Landfield, 1987; Khachaturian, 1994). It posits that dysregulation of calcium homeostasis is a primary factor contributing to aging-related learning and memory impairments observed in many species, including humans. We know that most of the neurodegenerative diseases involve serious calcium imbalances within their victims' brains. In general, people with any of these diseases experience an ever-worsening imbalance of calcium in their brain cells.

In healthy people, calcium ions are well managed, so they do not adversely affect neurons. However, in people with neurodegenerative diseases, the body gradually loses its ability to deal appropriately with excess calcium ions (Squier et al., 2000). CaBPs are important for regulating the intracellular calcium concentration of neurons (Baimbridge et al., 1992). In addition, studies have shown that neurons lacking in certain CaBPs are less able to handle various insults. For example, dissociated cells that are immunoreactive for the CaBP calbindin are better able withstand excitatory amino acid (EAA) insults, suggesting that the presence of calbindin (and perhaps other CaBPs) may serve important neuroprotective functions (Mattson et al., 1991).

In addition, numerous studies, including our own unpublished work (Moyer) suggest that there is a selective decrease in certain CaBPs in the brains of aged animals, including humans (Ichimiya et al., 1988; Iacopino and Christakos, 1990; Hof and Morrison, 1991; Amenta et al., 1994; Selden et al., 1994; Villa et al., 1994; De Jong et al., 1996; Zettel et al., 1997; Moyer et al., 2001). Loss of these important CaBPs with advancing age may leave certain populations of neurons vulnerable to any insult that results in massive or even moderate increases in intracellular calcium concentrations.

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With most major neurodegenerative diseases, there is an excess of unregulated calcium in the sensitive areas. As these diseases progress in their victims and the calcium imbalances worsen, the number of unregulated calcium ions in the nervous system increases. The body steadily loses its ability to control how calcium is stored and used. These unbound and excess calcium ions begin wreaking havoc on neurons. The unregulated calcium triggers excitotoxic events that will eventually kill the neurons. Currently, effective therapeutics that treat the problem of excess calcium ions are not available (Ripova et al., 2004).

Calcium-dependent processes have been shown to be important for associative learning in both adult and aged animals (Moyer et al., 1996; Thompson et al., 1996; Moyer et al., 2000). Also, compounds that block influx of calcium through L-type calcium channels have been shown to both improve associative learning in aged animals (Deyo et al., 1989; Disterhoft et al., 1993; Veng et al., 2003) and restore their electrophysiological properties to those commonly seen in young adults (Moyer et al., 1992; Moyer and Disterhoft, 1994; Thibault et al., 1998). Pharmacological manipulation of Ca²⁺ entry has been shown to be effective in increasing some aspects of cognitive function of the aged brain. Therefore, further exploration of Ca²⁺ homeostasis, regulation and signaling might reveal the mechanisms involved in the age-dependent decline in neuronal performance, and might aid the search for new therapeutic treatments. (Verkhatsky and Toescu, 1998, Pascale and Etcheberrigaray, 1999).

The relationship between calcium, neuronal degeneration, CaBPs, and aging suggest that a viable but as yet untapped treatment plan may involve replenishment of neuronal CaBPs, particularly in higher brain regions known to degenerate with advancing age, such as the hippocampus and associated MTL structures (Visser et al., 2002).

Neurodegenerative disorders:

Considerable evidence supports roles for calcium-regulating systems and altered calcium homeostasis in neurodegenerative disorders (Gibson and Peterson, 1987; Mattson, 1989; Khachaturian, 1989). Cellular degeneration is accompanied by impaired Ca(2+) homeostasis and a protective role for Ca(2+) binding proteins in certain neuron populations has been postulated (Heizmann and Braun, 1992). It has been assumed that neurons containing certain intracellular Ca(2+) binding proteins may have a greater capacity to buffer Ca(2+) and could be more resistant to degeneration (Scharfman and Schwartzkroin, 1989).

Disturbances in calcium homeostasis have been observed to be associated with Alzheimer's disease (AD) and other neurodegenerative diseases. Increased total calcium levels and reductions of calcium-binding proteins (calbindin-28k and calmodulin) have been found in AD brains (Sutherland et al., 1993). McLachlan et al. and Sutherland et al. discovered that in AD brains, the CaBP, calmodulin, was shown to be significantly diminished (McLachlan et al. 1987; Sutherland et al., 1993).

Huntington's disease (HD) is also dependent upon the correct functioning of the basal ganglia. The degeneration of neurons in the basal ganglia is thought to be due to excitotoxic activity related to an increase in intracellular calcium. As a result of this insult, increased levels of the calcium-binding proteins parvalbumin and calbindin D-28k has been noted in the assaulted areas (Parent et al., 1995; Huang et al., 1995). In Huntington's Chorea there is significant loss of calbindin-containing neurons in the basal ganglia (Seto-Oshima et al., 1988; Ichimiya et al., 1988).

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Parkinson's disease (PD) is also affected by a rise in intracellular calcium concentrations. The calcium-binding protein calretinin has been suggested as having a neuroprotective role in dopaminergic cell groups (Mouatt-Prigent et al., 1994). Decreases in calbindin-D28k in the basal ganglia have been found in cases of PD suggesting a neuroprotective function in the disease (Iacopino and Christakos, 1989). Calbindin containing subpopulations of neurons in the substantia nigra are spared in PD (Yamada et al., 1990) and in an animal model of the disease (Iacopino et al., 1992).

Aequorin:

One CaBP that might be effective in treating neurodegenerative disorders is aequorin, a naturally-occurring calcium-sensitive bioluminescent protein originally isolated from jellyfish (Inouye et al., 1985). Its natural bioluminescent properties (when bound to calcium) make it ideal not only for observing changes in calcium ion concentrations but also make it easy to observe uptake by neurons. Jellyfish have a very simple nervous system, and one function that aequorin appears to have in jellyfish is to sequester excess calcium ions by binding with them so they utilize the calcium for predation and self-defense.

Aequorin has been used as a calcium indicator for several decades in laboratory settings (Ridgeway, 1967).. The function of aequorin is unique from any other calcium-binding protein and has several distinguishing characteristics: Aequorin is non-toxic and does not interfere with internal cellular stoichiometry. (Miller et al., 1994) Each molecule of aequorin will bind with three calcium ions (Inouye et al., 1985). The protein is non-toxic when introduced into foreign cells (Blinks, 1990). Aequorin is considered as a 'relative' to the calcium-regulated effector protein calmodulin (Tsuji et al., 1995), which plays an important part in controlling a variety of cellular functions. Aequorin is from a family of CaBPs known as the EF-hand family. EF-hand proteins also constitute the other CaBPs endogenous to the human body. Aequorin and many of these endogenous proteins (i.e. calmodulin, parvalbumin, calbindin, calretinin) are homologous in structure and show strong nucleotide sequence similarity (Moncrief et al. 1990, Tsuji et al., 1995).

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Data:

Aequorin is taken up by hippocampal neurons. In a set of preliminary studies, aequorin was bilaterally injected directly into the hippocampus of 3 different adult rats. The rats were returned to their home cages for at least 24 hrs after which they were anesthetized and their brains removed, sectioned and stained for aequorin using a monoclonal anti-aequorin antibody. The primary antibody was then visualized using a secondary antibody conjugated to Alexa Fluor 594.

Figure 1 shows an example some aequorin-labeled hippocampal neurons using conventional fluorescence microscopy. The left panel is a photomicrograph of the hippocampus showing the the location of the cannula tip in the CA1 region. The white rectangle indicates the location of the two aequorin labeled CA3 pyramidal neurons shown in the right panel. The data clearly demonstrate that we are able to not only deliver aequorin directly into the brain but also that the aequorin spreads throughout the hippocampus and is then taken up by neurons in CA1, CA3, and the dentate gyrus. Our use of immunohistochemistry will enable us to not only determine the extent of aequorin spread throughout the hippocampus and neighboring structures, but it will also allow us to use dual labeling techniques to determine the types of cells (e.g., neurons vs. glial cells) that are actually taking up aequorin after just a single delivery or multiple deliveries.

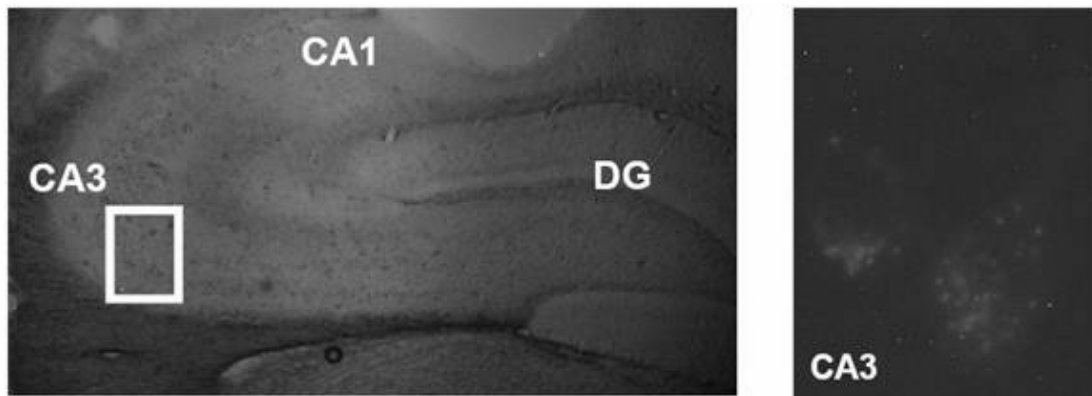


Figure 1. Aequorin is taken up by hippocampal neurons. Our preliminary data suggest that direct infusion of aequorin into the hippocampus (left panel) results in labeling of pyramidal neurons in CA1 and CA3. Aequorin (6% w/v) was dissolved in calcium-free aCSF in the presence of 3% DMSO. The solution was slowly injected (rate $\sim 1 \mu\text{l}/\text{min}$) directly into the hippocampus using a syringe pump (volume $\sim 1 \mu\text{L}$ per side). After completion of the injection, the delivery cannulae remained in place for about 1 min prior to removal. An example of two aequorin-containing CA3 pyramidal neurons is shown in the right panel (40X objective).

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