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Conference Theme

EXTREME BIOLOGY
LIFE IN DEEP SEA VENTS AND ANTARCTICA

Keynote Speakers

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Costantino Vetriani, Ph.D.
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Upregulation of Glutamate Transporter GLT-1 by AKT Signaling in Astrocytes Promotes Neuronal Survival in Alexander Disease

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Abstract

Alexander Disease (AxD) is a primary disorder of astrocytes caused by a missense mutation of an intermediate filament protein, GFAP. One pathological feature of AxD is the selective loss of hippocampal neurons accompanied by prominent protein aggregates within astrocytes. How a genetic deficiency of astrocytes leads to neuronal demise in AxD remains unknown. Uptake of glutamate (Glu) via the major glutamate transporter (GLT-1) of astrocytes is crucial for limiting Glu-mediated toxicity to neurons. Reduced GLT-1 expression and Glu uptake activity were present in a R236H GFAP knock-in mouse model and an established cell model of AxD. Accumulating evidence shows that Akt may induce the expression of GLT-1 through increased transcription in astrocytes. The present study is aimed to explore the relationship between GLT-1 and Akt in AxD. We found that phosphorylation of Akt on Ser473 and GLT-1 protein levels are coordinately decreased in an astrocytic cell line overexpressing R239C *GFAP* (the most common mutation in AxD). Reduced GLT-1 appears to be caused in part by the inactivation of Akt. Hippocampal neurons are more vulnerable to Glu-induced excitotoxicity when co-cultured with astrocytes expressing R239C GFAP. However, GLT-1 levels, GLT-1 currents, and neuronal protection can all be partially restored by expressing a constitutively active Akt. These observations demonstrate for, the first time, a potential pathogenic contribution of Akt-medicated GLT-1 dysfunction to neuronal loss in AxD.

Introduction

Intractable seizures, hippocampal neuronal loss and intracellular aggregates in astrocytes are common clinical and pathological features of Alexander disease (AxD), an autosomal dominant disease caused by a point mutation in the astrocyte gene encoding GFAP (Glial Fibrillary Acidic Protein)[1]. How an astrocytic encephalopathy caused by a mutation in GFAP compromises neuronal survival in AxD has remained an intriguing question for decades. AxD brain sections have markedly reduced glutamate type I transporter (GLT-1) levels[2]. R236H knock-in mice (corresponding to R239C mutation of human GFAP in AxD) also showed a dramatic reduction in GLT-1 protein in the hippocampus and increased vulnerability to kainate-induced seizures[3]. These observations are consistent with the notion demonstrated by GLT-1 deficient mice that defective Glu clearance by astrocytic GLT-1 results in a rise in extracellular Glu in the synaptic

cleft, excessive excitatory activity of neurons and high seizure susceptibilities[4,5].

Two main subtypes of Glu transporters (GluT) have been described in astrocytes of the mammalian forebrain and cerebellum: GLAST (EAAT1) and GLT-1 (EAAT2)[6]. Both can transit between the intracellular compartment and the membrane surface. Glu is transported by GluT and converted to glutamine within astrocytes via glutamine synthetase, then recycled back to neurons in the form of glutamine, which is hydrolysed to Glu within the mitochondria of synaptic terminals by phosphate-activated glutaminase, the glutamate–glutamine cycle shown in Fig. 1. GLT-1 is responsible for >90% of the total glutamate (Glu) uptake activity in adult forebrain[5]. The amount of GLT-1 protein which translocates from intracellular stores to the cell membrane is directly related to its Glu uptake activity, as measured by iGlu (Glu induced transporter current)[7].

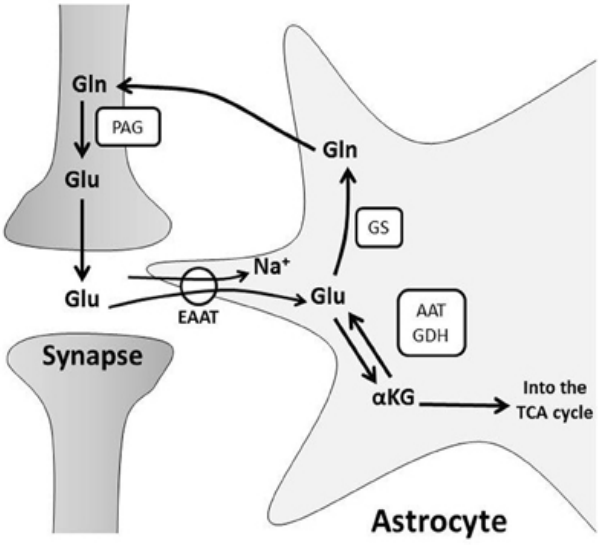


Figure 1. The glutamate-glutamine cycle

Glutamate (Glu) from pre-synaptic neurons stimulates post-synaptic neurons, and the signal is terminated by uptake of Glu from the synaptic cleft into astrocytes. Glu is primarily transported into astrocytes through Na^+-dependent excitatory amino acid transporters, EAATs. This disrupts the astrocyte Na^+ gradient and energy is consumed by the Na^+/K^+ ATPase to restore ionic concentrations. Glu is converted to: (a) glutamine (Gln) via glutamine synthase (GS) or (b) alpha-ketoglutarate (α-KG) by glutamate dehydrogenase (GDH) or aspartate aminotransferase (AAT) for subsequent oxidative metabolism in the TCA cycle. Gln is shuttled to neurons for glutamate production by phosphate-activated glutaminase (PAG) and the resulting Glu is repackaged in vesicles for further synaptic release[8].

We have previously shown a decrease in total and cell surface GLT-1 with diminished iGlu in astrocytes stably expressing R239C *GFAP*[2,9]. Moreover, primary astrocytes may selectively increase GLT-1 expression and GLT-1 mediated Glu transport via activation of proto-oncogene Akt (also called PKB, Protein Kinase B, a serine-threonine kinase as a general mediator of cell survival)[10-13]. Therefore we set out to examine whether Akt signaling is also involved in compromised GLT-1 function in AxD. We found severe reduction of GLT-1 protein accompanied by a concurrent loss of Akt Ser473 phosphorylation in R239C astrocytes, A constitutively active form of Akt (CA-Akt) boosted GLT-1 transcription, GLT-1 protein levels and transporter activity as assessed by iGlu, while a dominant negative form of Akt (DN-Akt) achieved the opposite effects. Moreover, boosting GLT1 expression with CA-Akt in R239C astrocytes partially restored ability of astrocytes to protect neurons from Glu-induced excitotoxicity.

Material and Methods

Electrophysiology

Whole-cell patch-clamp recordings from astrocytes have been described previously[2]. Briefly astrocytes were plated at 3 x 10^6 cells/coverslip in DM 3-4 days after 8BrcAMP differentiation, then were voltage clamped at a holding potential of -70 mV using the MultiClamp 700A patch-clamp amplifier with software MCC 700A Ver. 1.1.2.27 and pClamp 8 (Axon Instrument). Cell capacitance and series resistance was monitored throughout the experiments, and cells were discarded if the series resistance changed by >10%. The tip of the electrode that was used for puffing glutamate was about 2 μm in diameter. Other drugs for whole cell patch recordings were applied to the bath at final concentrations of: Dihydrokainic acid (DHK, 300μM), 6-nitro-7-sulfamoylbenzo [f]quinoxaline-2,3-dione (NBQX, 50 μM), (S)-α-Methyl-4-carboxy-phenyl-glycine (MCPG, 500 μM), 10 (RS)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid [(RS)-CPP] were purchased from Tocris (Ellisville, MO). Threo-β-hydroxyaspartic acid (THA, 250μM), and L-glutamate (1mM) was purchased from Sigma (St. Louis, MO).

Hippocampal cultures

Rat hippocampal cells (Sprague Dawley, P1) were seeded on polylysine-coated glass coverslips (6 x 10^4 cells/ coverslip) seated at the bottom of a 24 well tissue culture plate (Falcon) and maintained in neurobasal media with B27 supplement (Gibco-BRL) at 37°C. Proliferation of non-neuronal cells was inhibited by adding 5-FU (20 μM) at 20-40 h after plating for 72 h. The medium was exchanged every 2 days. Cells were used at 7-10d *in vitro* (DIV) unless stated otherwise.

Non-contact transwell cocultures

Astrocytes (RC2E10 cells stably expressing retroviral vector, retroviral-WT GFAP and retroviral-R239C GFAP, see Tian *et al*., 2010 for details) were trypsinized and transferred into a transwell insert (0.40 μm pores, 5x10^4/ insert membrane) sitting 1mm above the bottom layer of neurons growing in the 24 -well tissue culture plate. Thus the neurons and astrocytes are cultured in the same medium (Neurobasal+B27) for 24h, but are not in direct contact. For the induction of excitotoxic neuronal death, co -cultures were exposed to either 1mM of glutamate or 300μM of NMDA for 30min in a solution containing 20 mM HEPES, 120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 15 mM glucose, pH 7.4. After three extensive washes with control solution, cocultures were maintained for another 17 h before staining with anti-MAP2 and Hoechst 33258. Stained neurons and their nuclei were counted from 10 randomly selected fields of a 20x microscope objective per well. Control cultures were grown as above, but not exposed to glutamate or NMDA.

RT-PCR from astrocytes

Total RNA (0.5 μg) was extracted with Trizol (Gibco-BRL) from each group of astrocytes at 10 days after infection with Ad-Null, Ad-caAkt, Ad-dnAkt, respectively, followed by reverse transcription (15 min at 42 °C) and PCR-amplification (94 °C, 30 s; 62 °C, 30 s; 72 °C, 60 s) for the desired number of cycles (32 for GLT-1 and 18 for GAPDH) on a SmartCycler real -time PCR system (Cepheid, Calif.) according to the manufacturer's instructions. The following primer sequences were used for GLT-1 and GAPDH. GLT-1 (300 bp): 5'-TCTCCCTGTTGAATGAGACCATGA-3';

5'-TGCCTAGCAACCACTTCTAAGTCCT-3' (Pines, Danbolt et al. 1992); GAPDH (178 bp): 5'-TCACCACCATGGAGAA GGC- 3'; 5'-GCTAAGCAGTTGGTGG TGCA -3'.

Data analysis was performed with Cepheid software. Both the threshold cycle (C_T) and maximum fluorescence (FI) were used for interpretation of the results.

Antibodies

Primary antibodies included mouse anti-GFAP monoclonal antibody (mAb 3402; Chemicon), GLAST (AB1782, Chemicon) and GLT-1 (AB1783, Chemicon), SV40 Large T Antigen (554149, Pharmingen), GFP (A11122, Molecular probes; MAB3580, Chemicon). Rabbit anti-GLT1 and rabbit anti-GLAST antibodies for Western blotting were generous gifts from Dr. Michael Robinson (University of Pennsylvania) and Dr. Jeffrey Rothstein (Johns Hopkins University). All secondary antibodies were obtained from Southern Biotech.

Results

Reduced level of GLT-1 parallels a decrease of P-Akt in R239C astrocytes

Significant differences in the protein profiles of the control and R239C astrocyte cell lines were readily detected by Western blotting 24h after 8BrcAMP-induced differentiation (1mM for 4 days)[2]. GLT-1 protein level was significantly decreased in astrocytes expressing R239C GFAP (R239C), paralleling a marked reduction in phosphorylated Akt (p-Akt) at Ser473 (Fig. 2), relative to Ctrl (astrocytes stably expressing control vector) and WT (astrocytes stably expressing wild-type GFAP) cells. In contrast, neither GLAST nor total Akt changed appreciably in WT and R239C cell lines. All of these protein patterns remained similar from days 5 to 10 in the absence of 8BrcAMP (4 days of 8BrcAMP treatment referred to as day 0) (data not shown), suggesting that the changes of GLT-1 and p-Akt cannot simply be attributed to the effects of 8BrcAMP.

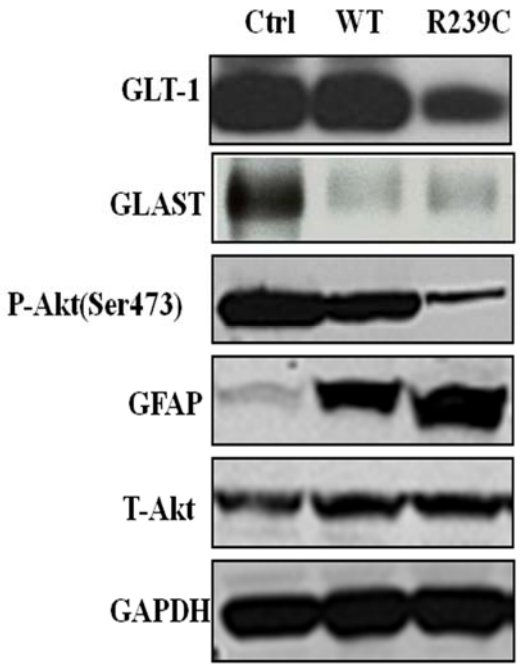


Figure 2. R239C astrocytes exhibit reduced GLT-1 and p-Akt level
Western blot analysis of endogenous GFAP, GLT-1, GLAST, Akt and p-Akt expression in an established cell model of AxD. Ctrl (Control) are astrocytes infected with retroviral vector backbone only; WT and R239C are astrocytes infected with retroviral vector expressing wild-type GFAP and R239C GFAP, respectively. GAPDH levels demonstrate equivalent loading of the lanes.

Akt-mediated GLT-1 upregulation improves GluT activity in R239C astrocytes

Since R239C expression resulted in a loss of GLT-1 and pAkt, we wanted to determine if these effects are linked. Each astrocyte line was infected with a control

adenovirus (VEC), an adenovirus expressing a dominant negative (kinase-dead) form of Akt (DN-Akt), or a constitutively active Akt (CA-Akt) resistant to PI3K/Akt inhibitors. Five days after adenoviral infection, LY294002 (LY, 20 μM), a potent and selective PI3K inhibitor, was added for 2 hr to attenuate the background Akt activation induced by adenoviral infection itself through the PI3K-Akt pathway[14-16]. All three cell lines were then analyzed to compare the relative GLT-1 mRNA (Fig. 3a) and protein levels (Fig. 3b). The CA-Akt produced a substantial increase of GLT-1 mRNA in each cell line, relative to the control adenoviral vector. In contrast, mRNA levels of GLT-1 were drastically lower in DN-Akt infected cells than those in control cells.

In general, the pattern of GLT-1 protein accumulation following CA- or DN-Akt overexpression resembled that of GLT-1 mRNA. Thus, high levels of p-Akt, produced by the CA-Akt adenovirus, correlated with high GLT-1 levels, while low levels of p-Akt, produced by the DN-Akt adenovirus, correlated with low GLT-1 levels (Fig. 3b, GLT-1 and p-Akt blots). These results were most marked in the astrocytes expressing R239C GFAP (Fig. 3b, lanes 7-9). Since the Akt constructs contained a small HA tag, we blotted all samples with an anti-HA antibody. This showed that levels of the exogenous Akt proteins were similar in all cells (Fig. 3b, HA), and that the results were therefore not biased by different exogenous Akt protein levels in different cells. Note that we observed a similar level of GLT-1 in WT astrocytes expressing the CA-Akt and Ad vectors (Fig. 3b, GLT-1, lane 4 and lane 6). Neither the level of HA-tagged CA -Akt (Fig.3b, HA, lane 4) nor a higher level of total p-Akt (Fig. 3b, p-Akt, lane 4 compared to lane 6) in WT astrocytes expressing CA-Akt can explain the above finding. A number of factors such as a better understanding of the turnover rates of GLT-1, other components of PI3K/Akt pathway, and the presence of Akt activation via pathways other than PI3K in our system have to be closely monitored in the future. The effects of Akt on GLAST protein were assessed in parallel. In accordance with the report that Akt can regulate GLT-1 expression with no effect on GLAST[17], we found that GLAST levels were comparable in all lines regardless of the treatment with different forms of Akt (data not shown). Considering that GLAST protein is relatively low in WT and R239C astrocytes (Fig. 2 GLAST), no further attempts were made to pursue this issue.

We also noted changes in GFAP levels, the most striking of which were found in R239C astrocytes (Fig. 3b, GFAP, lanes 7, 8 and 9). The CA-Akt induced a higher level of R239C GFAP (Fig. 3b, GFAP, lane 7), than the level in the R239C cells infected with the dn-Akt (Fig. 3b, GFAP, lane 9). The DN-Akt produced the lowest level of R239C GFAP (Fig. 3b, GFAP, lane 8). In contrast, levels of GFAP in Ctrl as well as in WT astrocytes were not affected by any of the adenoviral infections (Fig. 3b, GFAP, lane 1-3, 4-6). Although relatively little is known about the turnover rate of R239C GFAP, previous studies in our laboratory have demonstrated that autophagy, a mechanism by which accumulated proteins are cleared, is triggered by overexpression of R239C GFAP[18]. One consequence of activated Akt and its downstream target, mTOR kinase, is an inhibition of autophagy[19]. Hence it could be expected that activation of the Akt-mTOR pathway in R239C astrocytes would result in the inhibition of autophagy and consequently an increase in R239C GFAP level.

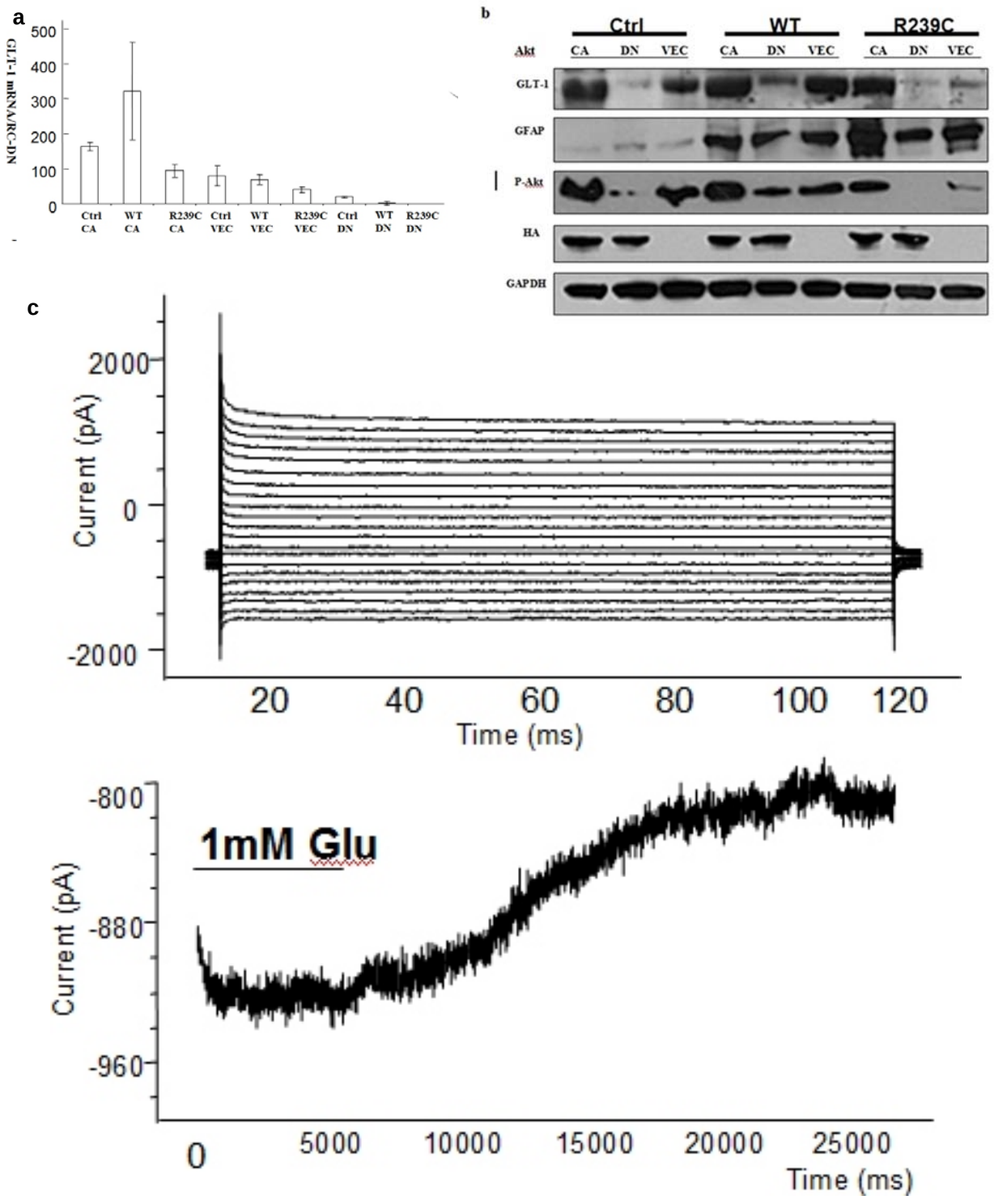


Figure 3. Regulation of GluT activity by Akt

Astrocytes were infected with an adenoviral vector expressing HA-tagged dominant negative (DN), or constitutively active (CA) and control gene (Ad vector, Ctrl) at a M.O.I. of 10 for 6 days (4 days of 8BrcAMP treatment referred to as day 0). Similar results were obtained in two other independent experiments. **a.** Quantitative Real-time RT-PCR analysis of the expression of GLT-1 mRNA. 5 μg of the total RNA extracted were reverse transcribed and PCR amplified quantitatively using primers specific for GLT-1, and GAPDH. The level of GLT-1 mRNA was normalized to GAPDH mRNA and is expressed relative to the amount observed in R239C astrocytes infected by DN-Akt. **b**. Levels of immunoreactive GLT- 1, HA-tagged Akt (CA-Akt and DN-Akt), p-Akt Ser-473, and GFAP relative to those of GAPDH in the total cell lysates. **c**. An iGlu similar to that in WT or Ctrl astrocytes was recorded in R239C astrocytes expressing CA-Akt.

Given the fact that activated Akt may act as a positive regulator of GLT-1 in astrocytes, it was important to determine if the increases in GLT-1 protein in the R239C astrocytes produced by the CA-Akt adenovirus resulted in an increase in GluT function (Fig. 3c). In contrast to the iGlu profiles in R239C astrocytes expressing control adenovirus, the average iGlu amplitude at –70 mV was increased by 47.5 ± 2.9% (iGlu in CA-Akt infected R239C, 35.2±5.6 pA; iGlu in control Ad vector infected R239C, 23.8±3.4 pA, $p<0.05$) and the number of responsive cells increased from 4 to 16 (+60%) in n = 20 experiments. Furthermore, the increased amplitude of iGlu observed above was effectively attenuated by 300 μM DHK, a selective GLT-1 uptake blocker (~ 60 - 70%, n = 7). This data provides strong evidence that the dramatic restoration of iGlu in R239C astrocytes expressing ca-Akt is predominantly a result of enhanced GLT-1 activity.

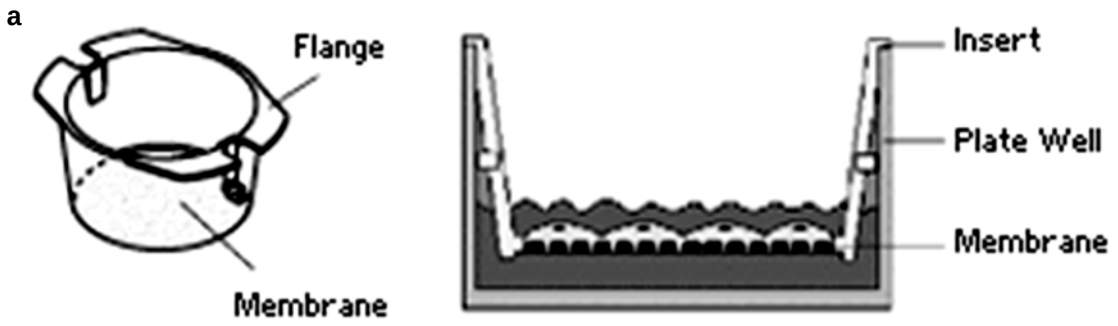
Akt expression in R239C astrocytes partially improves the survival of the hippocampal neurons.

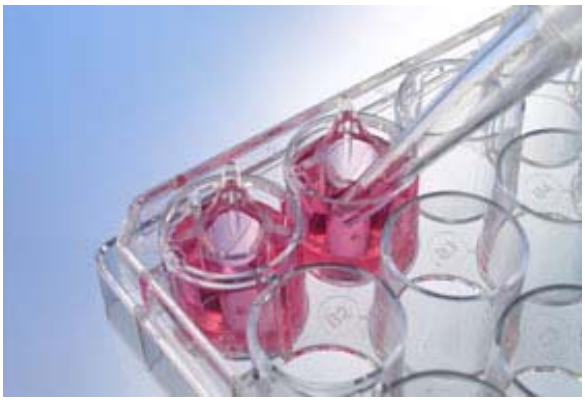
Integrating the above findings in the context of AxD, we decided to assay directly for cross-talk between astrocytes and hippocampal neurons in a transwell coculture system (Fig. 4a,b). We examined glutamate-induced hippocampal neuronal death in the presence of astrocytes expressing WT or R239C GFAP or astrocytes expressing the control retrovirus (VEC). Exposure to 1mM glutamate for 30 minutes led to a marked reduction in the numbers of MAP2+ neurons cocultured with R239C astrocytes, compared to the far better survival of neurons co-cultured with WT or control astrocytes .The impaired neuronal protection of R239C astrocytes was greatly reversed by infecting R239C astrocytes with the Ad-caAkt (Fig.4c, Ad-caAkt/Glu) and, to a lesser extent, with Ad vector only, The protection of the latter was probably due to induction of GLT-1 by adenovirus-mediated Akt activation (data not shown). Used as a control, NMDA (iGluR agonist) did not cause a significant reduction in the number of MAP2-positive neurons in the co-cultures (Fig.4c, NMDA), suggesting reduced neuronal viability by R239C astrocytes was prominently mediated by deficient GluTs. Additionally, it should be noted that the survival rate of neurons co-cultured with R239C astrocytes (14% with Glu) was dramatically reduced compared with those cultured with control astrocytes (61.9% with Glu). One possible explanation is that R239C astrocytes could release neurotoxic molecules (Glu or cytokines, Wu *et. al*. personal communication) after 1mM Glu treatment[20].

Discussion

Our study indicates at least three key players critically involved in the reduced GLT-1 capacity in astrocytes expressing the R239C mutation of GFAP. Expression of the earliest player, R239C GFAP itself, results in a marked reduction in the second player, p-Akt, which in turn down-regulates the transcription and translation of the third player, GLT-1.

R239C GFAP is the most common AxD mutation[10]. Like other cytoskeletal components, GFAP is in dynamic equilibrium between assembled filaments and unassembled subunits. *In vitro* assembly studies have shown that R239C GFAP forms 10 nm intermediate filaments indistinguishable from WT filaments under electron microscopy[21]. Immuno-fluorescence microscopy of cultured cells,





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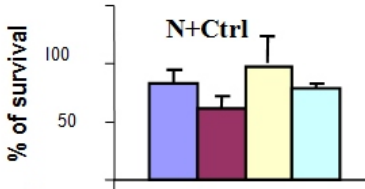

Average %			
NMDA	83.3 %	66.6%	57.8%
Glu	61.9%	48.3%	** 14.7%
Ad-caAkt/Glu	98.3%	93.5%	* 69.6%
Ad/Glu	78.4%	88.2%	* 48.2%

Figure 4. R239C astrocytes do not protect neurons from Glu-toxicity, but activation of Akt partially restores protection

Hippocampal neurons were prepared and maintained for 7-10 days in vitro (lower chamber) and co-cultured with VEC, WT or R239C astrocytes (upper chamber) for 24hr, as illustrated in 4a, the transwell coculture system. Then either L-glutamate (Glu, 1mM) or NMDA (300 μM) was added to the medium for 1h (4b). Neuronal viability in each group (N+Ctrl: co-cultured with vector control astrocytes; N+WT, co-cultured with WT astrocytes; N+R239C, co-cultured with R239C astrocytes) was determined 17h later by counting total intact nuclei of MAP2+ neurons for 10 random fluorescence fields (20x) per experiment (normalized to cocultures in the absence of glutamate). Neuronal death following Glu exposure was indicated by Hoechst33258 stainings showing condensed, fragmented, nuclear and chromatin changes. A summary is charted in (c). Ad-caAkt/Glu: Astrocytes infected with the Ad-ca-Akt vector; Ad/Glu: Astrocytes infected with the control adenoviral vector. The data presented are the mean ± SD of triplicate determinations. *, $P < 0.05$; **, $P < 0.01$.

however, reveals that R239C GFAP assembles into disorganized filament bundles and/or aggregates, which appeared to disrupt the normal filamentous architecture of WT GFAP in human astrocytes[22]. What remains unclear is how the mutation affects the intrinsic property of GFAP organization via disassembly and reassembly, although it seems likely that the association of GFAP with increased levels of αB-crystallin, a small heat shock protein, and plectin, a cytoskeletal linker protein, represents failed attempts of astrocytes to debundle or disaggregate the R239C GFAP[22, 23].

The second player, pAkt, is significantly lower in R239C astrocytes than in control cells or astrocytes that express WT GFAP. Akt activation is a complex process consisting of at least two sequential steps, namely the membrane translocation and the subsequent phosphorylation of Akt at Ser473 and Thr308[24, 25]. Therefore, the activation of Akt could be disturbed at either step, which cannot be distinguished by forced membrane targeting of CA-Akt (myristoylated) in our study. However, it has been demonstrated that Akt translocates from cytosol to the plasma membrane often near or at cell–cell or cell–matrix contacts. A simple yet logical explanation to the Akt inactivation is that R239C aggregates might sequester the cytosolic Akt away from its normal site of action[26]. Another possibility arises from accumulating evidence that disruption of gap junctional communication results in reduced PI3K/Akt activation[27-29]. In favor of such a possibility, we found R239C astrocytes are poorly coupled. Clearly, additional experiments including immunoprecipitation, subcellular fractionation, Akt kinase assays and examination of intercellular $Ca2^{+}$ waves are required to address the molecular links between GFAP mutations and Akt inactivation.

The down-regulation of GLT-1 transcription and translation mediated by inactivation of Akt is evident in the R239C astrocytes. However, the fact that CA-Akt is not sufficient to restore iGlu completely suggests that decreased transcription/synthesis cannot be the sole contributor to GLT-1 dysfunction. Other possibilities may include altered splicing, post-translational modifications, accelerated turn-over rates, impaired cytosolic trafficking as well as mistargeting to the microdomains of plasma membrane[30]. Nevertheless, our finding represents an essential step for further in-depth characterization of GluTs function in AxD.

Intercellular miscommunication in AxD

Results in the neuron-astrocyte co-culture system suggest a connection between astrocyte dysfunction and neuronal demise in AxD. First, the ability of GluT-deficient R239C astrocytes to protect neurons against Glu excitotoxicity is compromised. Second, expression of CA-Akt in R239C astrocytes, which significantly restores the GluTs, improves the survival of neurons following Glu exposure. In addition to aggregates-bearing astrocytes and selective neuronal loss, another pathological feature of AxD is marked loss of oligodendrocytes, the myelin-producing cell of the CNS. Interestingly, excitotoxicity caused by sustained high levels of extracellular Glu damages not only neurons, but also oligodendrocytes[31-34], leading to the possibility that reduced capacity of the

GluTs on astrocytes may also contribute to axonal dysfunction and/or death of oligodendrocytes in AxD.

Acknowledgments

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Presence of Octopamine and an Octopamine Receptor in *Crassostrea virginica*

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Abstract

Octopamine is a biogenic amine first identified in octopus. It has been well studied in arthropods and a few gastropods, serving as a neurotransmitter and hormone. The presence of octopamine has rarely been reported in bivalves and has not been reported in *Crassostrea virginica.* We utilized HPLC to identify and measure octopamine in cerebral ganglia, visceral ganglia, gill, palps, mantle, heart and hemolymph of *C. virginica.* Endogenous octopamine levels increased when animals were treated with tyramine, an octopamine precursor. A preliminary study in our lab found that octopamine has a cardio-excitatory action on *C. virginica* heart. In the present study we also used immunoblotting techniques to identify an octopamine-like receptor (Pan TAAR, trace amine-associated receptor) in oyster heart. The study confirms the presence of octopamine in the nervous system, innervated organs and hemolymph of *C. virginica* and identifies the presence of an octopamine-like receptor in heart, strengthening the contention that octopamine is important in the physiology of *C. virginica* as it is in other invertebrates.

Introduction

Studies of the nervous system of bivalve molluscs show the biogenic amines serotonin, norepinephrine and dopamine to be present and serve as neurotransmitters and neurohormones[1-8]. Many of these studies focus on the physiology of the heart and gill. Hearts of different bivalve species tend to have different responses to various neurotransmitters. Bivalve heart tends to be inhibited by acetylcholine and in most species excited by serotonin[9,10]. Biogenic amines are present and have neurophysiological functions in *Crassostrea virginica*[11-14]. Dopamine and norepinephrine are involved in stress responses of *C. virginica* and endogenous dopamine and norepinephrine levels increased in response to mechanical stress[15,16]. Temperature or salinity changes also increase dopamine and norepinephrine levels in bivalves[17,18].

Octopamine is a biogenic amine that was first identified in salivary glands of octopus[19]. It has been well studied in gastropods and insects where it serves as a neurotransmitter and hormone[20-24]. It has been less well studied in bivalve molluscs[25]. In the clam *Tapes watlingi* octopamine was found to have an excitatory action on the isolated, spontaneously beating heart[26] and octopamine receptors were identified in the animal's accessory ventricle[27]. A preliminary study in our lab found that octopamine has a cardio-excitatory action on *C. virginica* hearts[28]. The present

study sought to determine if octopamine and an octopamine receptor were present in the heart and other tissues of *C. virginica*.

Materials and Methods

Adult *C. virginica* of approximately 80 mm shell length were obtained from Frank M. Flower and Sons Oyster Farm in Oyster Bay, NY, USA. They were maintained in the lab for up to two weeks in temperature-regulated aquaria in Instant Ocean artificial sea water (ASW) at 16 - 18°C, specific gravity of 1.024 ± 0.001, salinity of 31.9 ppt, and pH of 7.8 ± 0.2. Each animal was tested for health prior to experimentation by the 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 used for the experiments. Octopamine hydrochloride, tyramine, and 1-octanesulfonic acid (sodium salt, Sigma Ultra) were obtained from Sigma-Aldrich (St. Louis, MO, USA. For HPLC analysis. Gemini 5μ C18 reverse phase HPLC columns were obtained from Phenomenex (Torrance, CA). For immunoblotting analysis, NP-40 lysis buffer, Bradford reagent, Laemmli 2X loading buffer containing βME, Bio-Rad Mini-Protean TGX gels, Bio-Rad Precision Plus Protein WesternC Standards, Tris/ glycine SDS buffer and Bio-Rad Precision Protein StrepTactin-HRP conjugate were obtained from Bio-Rad. Western Blot Signal Enhancer was obtained from Pierce. Pan TAAR (trace amine-associated receptor) 1° antibodies (goat polyclonal, sc-54398), and polyclonal HRP-conjugated 2° antibodies (chicken anti-goat, sc2953) were obtained from Santa Cruz Biotechnology. CN/DAB Substrate and all other reagents were obtained from Fisher Scientific (Pittsburgh, PA, USA).

HPLC Analysis of Tissues

Oyster tissues (cerebral and visceral ganglia, gill, palps, mantle and heart) were excised, blotted and weighed. Approximately 1 gram of each tissue was homogenized in 2 ml of 0.4 M HCl with a Brinkman Polytron homogenizer with Omni International disposable probe tips. One ml of hemolymph was drawn from adductor muscle with a syringe and mixed with 1 ml of 0.4 M HCl. The samples were centrifuged (15,000 x g, 20 minutes) and resulting supernatant vacuum filtered through 0.24 micron filters. Tissue filtrates were analyzed for endogenous octopamine levels using HPLC with fluorescence detection[12]. Samples (20 μl) were injected into a Beckman System Gold 126/168 HPLC system fitted with a Phenomenex-Gemini 5μ C18 reverse phase, ion pairing column with a guard column. All reagents were HPLC grade. The acetate/methanol (85:15 v/v) mobile phase (50 mM acetate buffer, pH 4.7 containing 1.1 mM of 1-octanesulfonic acid and 0.11 mM EDTA) with a flow rate of 2 ml/min in isocratic mode. A Jasco FP 2020 Plus Spectrofluorometer fitted with a 16 μl flow cell was used for detection of native fluorescence (280 nm excitation, 320 nm emission). Octopamine levels were quantified by comparing the peak areas of samples to those of standards and are reported as ng/g wet weight for tissues (gill, palps, heart and mantle), ng/ ml for hemolymph and ng/ganglion for both cerebral and visceral ganglia. Statistical analysis was determined by a t Test.

Western Blot Analysis for Heart Octopamine Receptor

Three oyster hearts were dissected, rinsed well in ASW, blotted and weighed, and placed in eppendorf tubes with 2.5 ml of ice cold NP-40 lysis buffer containing protease and phosphatase inhibitors. Each tube was sonicated on ice for 2-3, 5 sec bursts with a Brinkman Polytron and then kept on ice for 30 min before being centrifuged (10,000 x g for 20 min). The resultant lysate supernatants were pooled and aliquots were analyzed for protein concentration by Bradford assay. The remaining lysate supernatants were adjusted to a protein concentration of 4-5 mg/mL for SDS PAGE.

Preparation of Samples for Loading into SDS-PAGE Gels

Aliquots of lysate protein were denatured with Laemmli 2X loading buffer containing βME (1:1 ratio) and allowed to sit for one hour at room temperature. Laemmli-treated samples (20-40 μg total protein) were wet-loaded into wells of polyacrylamide gels (Bio-Rad Mini-Protean TGX gels), alongside pre-stained molecular weight markers (Bio-Rad Precision Plus Protein WesternC Standards). Gels underwent electrophoresis in Tris/glycine SDS buffer for 1 hour at 150 v.

Western Blot Analysis

After electrophoresis, gels were removed from their plate, rinsed in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol, pH 8.3), and sandwiched for transfer onto nitrocellulose membranes. Before immunoblotting was started, pre-stained markers were visualization to access whether proteins migrated uniformly and evenly during the electrophoresis process. The wet-transfer was done in a Mini Trans-BlotR electrophoretic transfer cell (Bio-Rad) under constant current (20 v) for 150 min in the presence of a cooling module to prevent excess heating. After transfer the membranes were rinsed with ddH_2O, treated with a Western Blot Signal Enhancer, rinsed 5x with ddH_2O, and then blocked with 5% non-fat dry milk in TBS-T for one hour at room temperature. After blocking, membranes were incubated at 4°C with 1° antibody (Pan TAAR at 1:400 dilution). Pan TAAR is reactive with octopamine, beta-phenylethylamine, p-tyramine (p-TYR) and tryptamine receptors, but unresponsive to classical biogenic amines (serotonin, dopamine, norepinephrine and epinephrine) and histamine receptors. The Pan TAAR 1° antibodies were diluted in TBS-T and 2% blocker for 24 hours, then the membranes were washed extensively with TBS-T, followed by incubation at room temperature for 60 min with HRP-conjugated 2° antibody (1:4000 dilution) in TBS-T and 2% blocker, and Bio-Rad Precision Protein StrepTactin-HRP conjugate (1:5000 dilution) was used to resolve protein standards. After incubation the membranes were washed extensively with PBS-T and chromogenic detection of HRP-conjugated standards and lysate proteins were resolved using CN/DAB Substrate. After the protein blots were chromogenically developed, the images were captured with a Carestream GI212 Pro Molecular Imaging System.

Results and Discussion

Reports of octopamine presence in peripheral tissues of bivalves or gastropods are scarce, beyond the few reports showing it to be present in heart

and hemolymph[23,29,30]. In this study we looked for octopamine using HPLC and found octopamine to be present in hemolymph and other peripheral tissues of the oyster *C. virginica*. This HPLC procedure with fluorescence detection showed an octopamine retention time of 4.1 minutes and was able to detect octopamine standards at amounts lower than 10 pg. The standard curve was fairly linear up to about 2 ng (Fig. 1). The identification of octopamine from tissue samples was confirmed by identical retention time and spectral characteristics compared to octopamine standards (Fig. 2a). Co-injecting octopamine standards with gill tissue samples (Fig. 2b) or hemolymph samples (Fig, 2c) revealed a single octopamine peak with the same 4.1 minute retention time. Octopamine was present in all *C. virginia* tissues samples tested (Fig. 3). Tissue amounts were in the 300 ng/g range for gill, palps, heart and mantle, while hemolymph had about 20 ng/ml. Our results with *C. virginica* compares well with other reports of octopamine, which was present in the hearts of the snail, *Helix* and the clam, *Tapes,* at about 4X and 1/4 as much, respectively[30,31]. Since tyramine is a direct precursor to octopamine, in other experiments gills were incubated for 24 hours with 5 mM tyramine to determine if the treatment would result in an increase in endogenous octopamine levels. HPLC analysis of tyramine treated gills found an octopamine peak representing more than a 10 fold increase in endogenous octopamine concentration compared to controls (Fig. 4).

Compared to peripheral tissues, there are numerous reports of octopamine in nerves and ganglia of gastropods[23], and even a few in nerves and ganglia of bivalves[25]. Our HPLC results found that the cerebral and visceral ganglia of *C. virginica* had approximately 1 ng/ganglion each (Fig 5). Octopamine amounts in ganglia of snails and nudibranchs, which are much larger than the ganglia of *C. virginica*, were reported to be about 10 to 100 times greater[29].

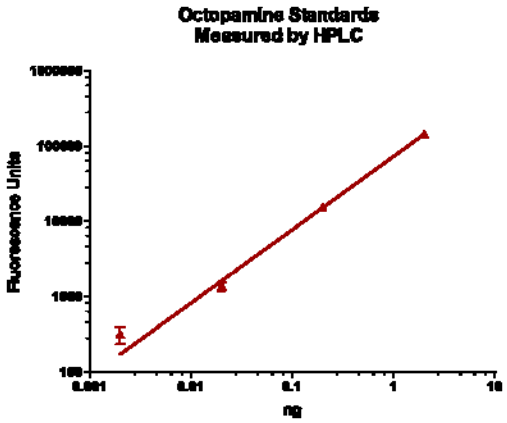

Fig. 1. Standard curve of octopamine standards analyzed by HPLC HPLC with native fluorescence detection (280 nm excitation, 320 nm emission). X and Y axis are log scale.

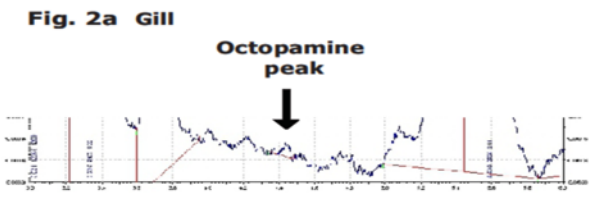

Fig. 2a. Record of HPLC analysis of octopamine from gill.

Earlier preliminary experiments in our lab showed octopamine has a physiological effect on oyster heart, and that could be blocked by the octopamine antagonists phentolamine and metoclopramide. In view of the fact that the present HPLC results found that oyster heart contains a significant amount of octopamine (270 ng/g wet weight), we used Western Blotting to look for the presence of an octopamine-like receptor in heart tissue. Using the Pan TAAR

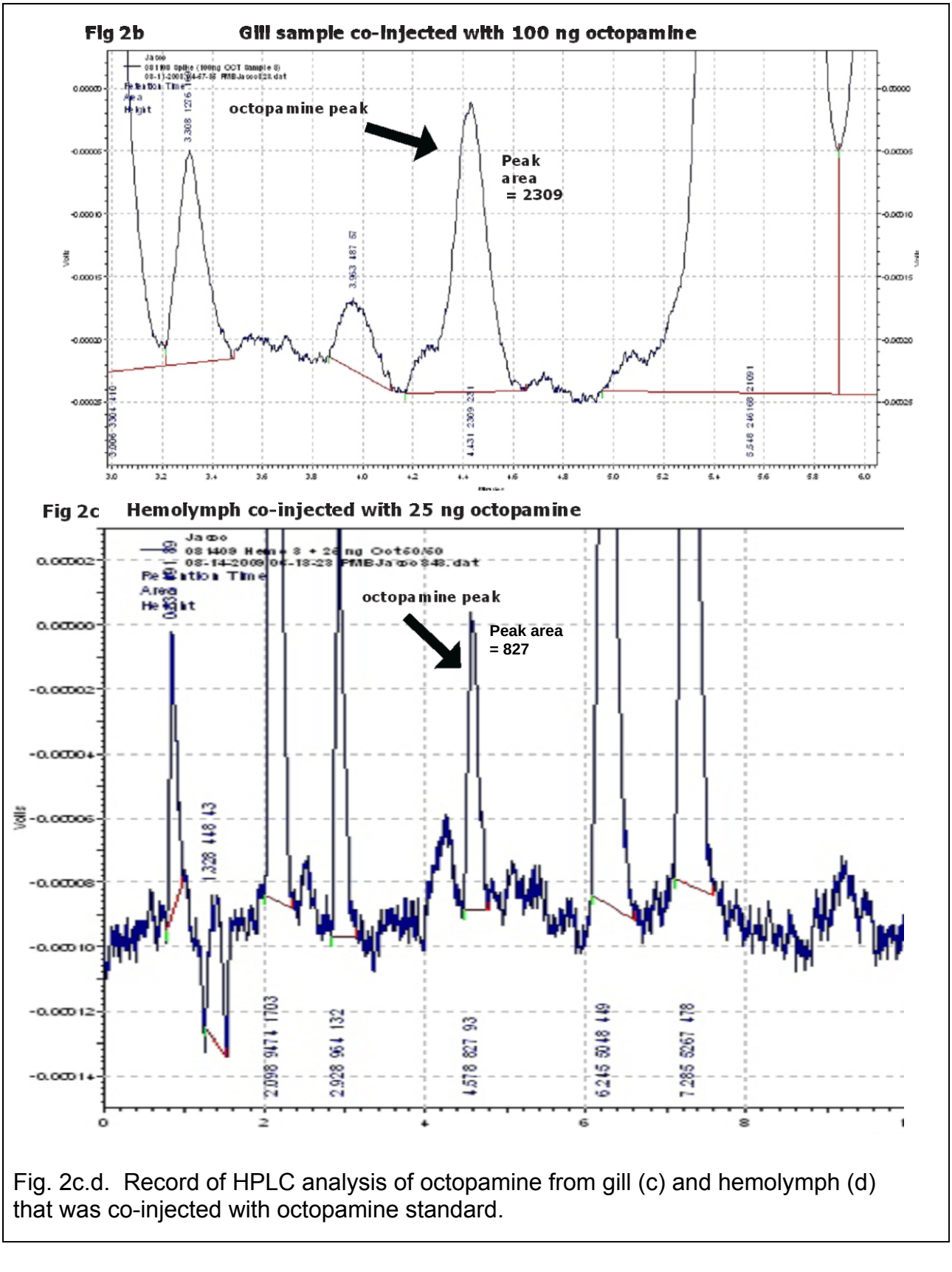

Fig. 2c.d. Record of HPLC analysis of octopamine from gill (c) and hemolymph (d) that was co-injected with octopamine standard.

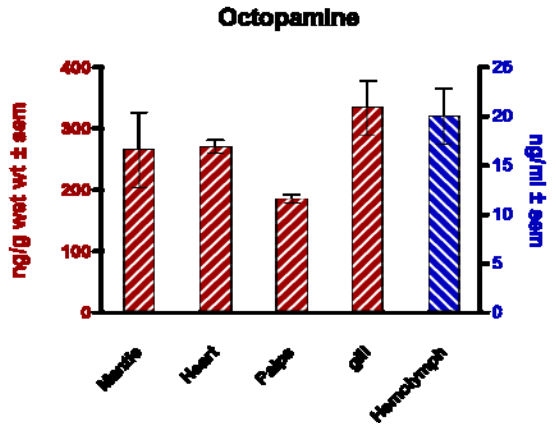

Fig. 3. Octopamine levels in peripheral tissues and hemolymph determined by HPLC analysis.

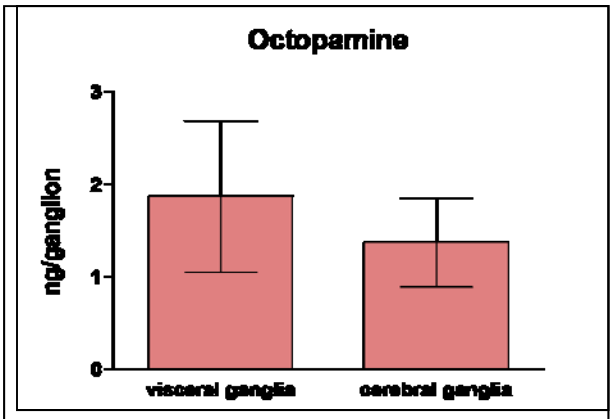

Fig. 5. Octopamine levels in visceral and cerebral ganglia determined by HPLC analysis.

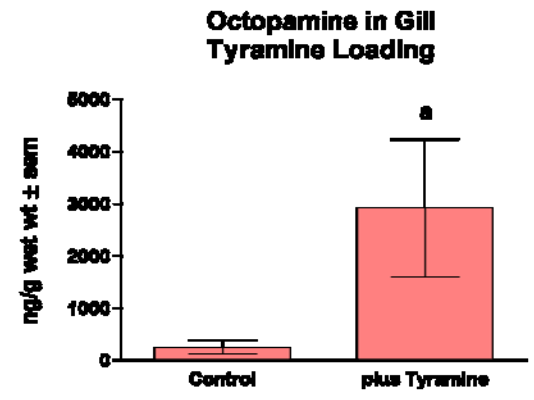

Fig. 4. Octopamine levels in gill incubated with 5 mM of tyramine for 24 hours. Statistical analysis was determined by a t-Test. $^{a}p < 0.05$ compared to controls.

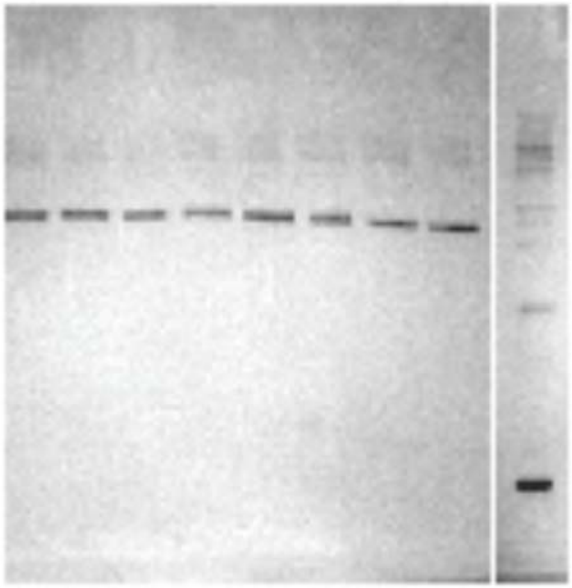

Fig. 6. Western Blotting of heart tissue showing a strong protein band at about 80 kD indicating the presence of an octopamine receptor. Pan TAAR 1° antibodies (goat polyclonal) and HRP-conjugated 2° antibodies were used. The right lane contains the protein markers.

antibody, which binds to octopamine receptors, our Western Blot revealed a single strong protein band at approximately 80 kD (Fig. 6) in agreement with Farooqui *et al*[32] who found an octopamine receptor band from honeybee brain at 78 kD. Octopamine receptors also have been identified in *Tapes* clam heart[27].

The study identifies the presence of octopamine as an endogenous biogenic amine in *C. virginica*, and shows that *C. virginica* has the enzymatic pathway to synthesis it from its precursor tyramine. The study also confirms our previous pharmacological work that suggested a possible physiological role of octopamine as a cardio-excitatory agent by identifying the presence of an octopamine-like receptor in *C. virginica* heart. Based upon these results we will conduct a full physiological study of the effects of octopamine on oyster heart as well as a comparative study of octopamine and octopamine receptors in other bivalves.

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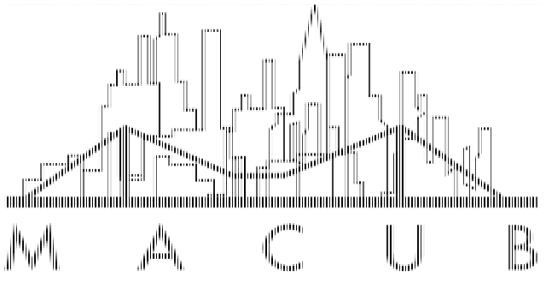

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