CDP-CHOLINE NEUROPROTECTION AND VASCULAR REMODELLING VIA IRS-1 MECHANISMS IN VASCULAR DEMENTIA

GROWING NEURONS ATOP MICROELECTRONIC CHIPS AS A NEW MODEL FOR NEURODEGENERATION

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ABSTRACT

Over the past couple of decades there have been no major improvements in the treatment of either acute ischaemic stroke or one of its major complications, neurodegenerative dementia. The majority of promising clinical trials associated with attempts to protect against the debilitating effects of Alzheimer's disease have ended prematurely following exasperation of the condition. Citicoline has been used extensively in patient trials following stroke but strong evidence for its ability to enhance neuroprotection and improve recovery are still lacking. Following our demonstration that this molecule can also enhance angiogenesis/vascularization we hypothesized that it could benefit patients suffering from vascular dementia. Here we describe our recent findings, discuss citicoline signalling and possible links to neurovascular degradation/protection, and consider novel mathematical-biological systems methodology for the identification of potential protective/therapeutic agents against this disease.

ملخص: على مدى العقدين الماضيين لم يكن هنالك أي تطور كبير في علاج السكتة الدماغية الحادة أو احد مضاعفاتها الرئيسية وهو الخرف الناتج من تلف الاعصاب وغالبية التجارب السريرية الواعدة المرتبطة بمحاولات الحماية من الآثار المدمرة لمرض الزهايمر توقفت قبل اوانها. استخدم السيتيكولين على نطاق واسع في التجارب علي مرضي السكتة الدماغية ولكن لا توجد براهين قوية علي قدرته على تعزيز الحماية العصبية وتحسين الشفاء. حسب تجربتنا فيمكن لهذا الجزيء ان يعزز تكوين الأوعية الدموية مما يؤدي للافتراض بأنه يمكن أن يستفيد منه المرضى الذين يعانون من الخرف الوعائي. سنوصف هنا النتائج التي توصلنا إليها مؤخرا، وسنناقش إشارات السيتيكولين والروابط الممكنة لحماية الاوعية الدموية للأعصاب من التلف، كما سننظر في منهجية النظم الرياضية البيولوجية لتحديد العوامل الوقائية و العلاجية المحتملة ضد هذا المرض.

Received: 20 December, 2012; Accepted: 15 February, 2013
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INTRODUCTION

Evidence of reduced blood-brain barrier (BBB) integrity preceding other Alzheimer's disease (AD) pathology provides a strong link between cerebrovascular pathology and AD. In animals models, amyloid-β peptide - injected animals exhibited a commonality in perturbations of microvessels compared with those evident in AD brain[22]. It was suggested that amyloidogenesis promotes extensive neoangiogenesis leading to increased vascular permeability and subsequent hypervascularization in AD. In human patients hypervascularity was corroborated in a comparison of postmortem brain tissues from AD. Brain microvessels derived from patients with AD expressed numerous factors implicated in vascular activation and angiogenesis. Signaling cascades associated with vascular activation and angiogenesis were upregulated in AD-derived brain microvessels[21]. However, these newly formed blood vessels may be non-functional. All above provides a new paradigm for integrating vascular remodeling with the pathophysiology observed in AD[20]. Therefore, vascular activation hypothesis could be a novel, unexplored therapeutic target in AD.

Background of our own recent investigations: In our recent study, we have, for the first time, demonstrated both a vascular protective, and proangiogenic effect of citicoline using *in vivo* and *in vitro* models[19]. Our data suggests a strong protective effect against the damaging process of excitotoxicity and hypoxia, similar to that experienced after acute ischaemic stroke. In regard to the possible mechanism our protein studies demonstrated that citicoline induced pERK1/2 expression, a key mitogenic signalling protein known to be involved in angiogenesis and generally stimulated by growth factors through interaction with their receptors[23].

There is the potential of citicoline to activate intra-cellular signal transduction pathways and induce phosphorylation of down-stream angiogenic molecules; hence we investigated this ability in more detail by analysis of the Kinexus-phospho-protein Western screening following treatment of vascular EC with citicoline.

Interestingly, treatment with citicoline modified the expression of only several of the >500 proteins on the array showing a degree of specificity. Insulin receptor substrate-1 (IRS-1) was phosphorylated in the presence of citicoline. IRS-1 over-expression was attributed to increased angiogenesis in human EC in association with increased Akt and VEGF-A expression [Stephens et al, 2012], whilst *in vivo*, antisense IRS-1 sequences delivered by sub-conjunctival injection inhibited rat corneal neovascularisation[18], and when delivered by means of eye-drops (GS-101) were found to be tolerable in a phase-1 clinical trial and may be sufficient to prevent neovascularisation in disease such as retinopathy and neovascular glaucoma [15].

Therefore, IRS-1 represents a potent modulator of pro-angiogenic signalling cascades in vascular EC and as such, since we have shown both *in vitro*, and in the rat model of temporary MCAO that citicoline induces phosphorylation of IRS-1 and concomitant EC activation and increased vascularisation. This could be a key novel mechanism of action of citicoline implicated in stroke recovery pathways and angiogenesis until now. This may be an extremely valuable novel finding in regard to understanding the potential mechanisms through which citicoline treatment results in patient recovery, since both protection of EC and induction and maintenance of angiogenesis is key to both short-term and

chronic re-vascularization after stroke impacting indirectly but significantly also on neuronal survival and re-integration[17]. Figure one for details[19].

IRS-1 in neurodegeneration: Only recently, it has been demonstrated that beta-amyloid (Abeta) oligomers are implicated in Alzheimer's disease leading to phosphorylation and degradation of the adaptor protein insulin receptor substrate-1 (IRS-1). IRS-1 couples insulin and other trophic factor receptors to downstream kinases and neuroprotective signaling. Increased phospho-IRS-1 is found in AD brain. Levels of IRS-1 and their activated kinases correlated positively with those of oligomeric Aβ plaques and are negatively associated with episodic and working memory, even after adjusting for Aβ plaques, neurofibrillary tangles, and APOE ε4. Brain insulin resistance thus appears to be an early and common feature of AD, a phenomenon accompanied by IGF-1 resistance and closely associated with IRS-1 dysfunction potentially triggered by Aβ oligomers and yet promoting cognitive decline independent of classic AD pathology[17,18]

New assays for neuronal degeneration: It is now understood that periodic behaviour is not confined to a limited number of physiological processes but is abundant in most biological systems. Periodicity in processes of the human body encompass phenomena such as genetic interactions, heartbeat rhythms, oscillating secretory, retina and muscle cells, cytoskeletal structures, bacterial oscillations, rhythmic behaviour in growth and development, and most importantly for this study, neuronal oscillations[1-3]. In 1952, whilst modelling neurons, Hodgkin and Huxley were able to accurately model the action potential in the giant squid axon[4]. Their nonlinear ordinary differential equations approximate electrical characteristics of excitable oscillatory cells such as cardiomyocytes and neurons. In 2009, Borresen and Lynch[5] published a paper suggesting a novel idea based on biological neural computing utilising the Hodgkin-Huxley equations, and following this work, three years later UK and International patents were published[6]. The patent illustrates how it is possible to construct binary logic gates from biological neurons grown on a chip. It is proposed that neurons could be grown on a chip and that they could be trained to perform certain logical operations. Binary oscillator logic gates could then be used to test drugs that may halt or reverse symptoms of neurological disorders such as Alzheimer's, Parkinson's disease and epilepsy. A number of research groups are

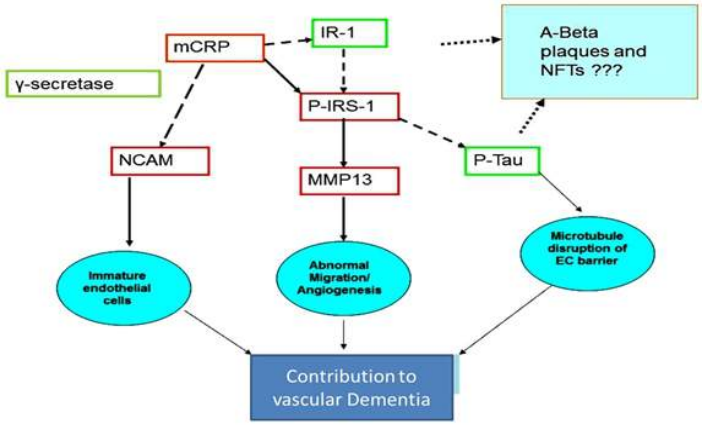

Figure 1 shows the operation of a binary half-adder based on Fitzhugh-Nagumo oscillators (which are simplified versions of the Hodgkin-Huxley models).

now able to grow neurons on chips using a variety of techniques including an aligned micro-contact printing technique, patch clamping (which yields very accurate information but is invasive) and extracellular recordings by means of external micro-transducers or optical measurements (which are non-invasive)[8-13], and it has recently been shown that Parkin diseased neurons can also be grown on a chip[14]. In 2012[15], it was shown that citicoline could be used as a protective treatment against Alzheimer's following a stroke.

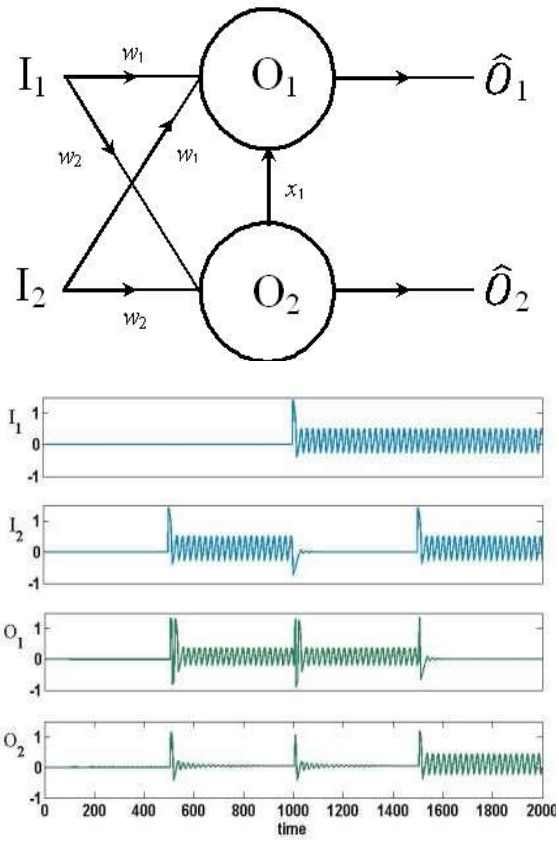

Figure 2: (a) Schematic of a binary oscillator half-adder. (b) Numerical simulation: Time series showing correct logic function of a half-adder. An oscillation corresponds to a 1 in binary and no oscillation is a zero. The sum oscillator O_1 will oscillate if either I_1 or I_2 is active. The carry oscillator O_2 will oscillate if both I_1 and I_2 are active. An inhibitory connection, say x_1, from O_2 to O_1 suppresses oscillator O_1 if O_2 is active.

Based on our previous results and citicoline involvement in blood vessel remodelling via IRS-1 pathway together with a new evidence of vascular hypothesis in AD, we aim to develop an *in vitro* assay for neuronal degradation of Alzheimer's diseased neurons. In this model we will study effects of citicoline/IRS-1 pathways on neurones grown on atop microelectronic chips in conditions of ischemia and neurodegeneration. It is a multidisciplinary project drawing on Medicine, Biology, Mathematics, Chemistry, Physics, Engineering and Computing.

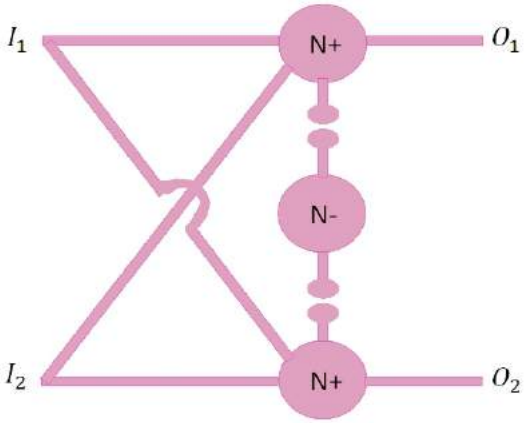

Figure 3: Schematic of a binary oscillator half-adder using biological neurons. Neurons N+ depict excitatory neurons and neuron N- depicts an inhibitory interneuron. Using the logic circuitry highlighted by Lynch et al[6,7,16], we believe it is possible to build an assay for neuronal degradation. Implementation of the half-adder using biological neurons on a chip will require three neurons as neurons are either excitatory or inhibitory, they cannot be both. Figure 3 shows a schematic of the biological half-adder

FUTURE DEVELOPMENTS

We have already shown that it is possible to construct logic gates and memory using threshold oscillator logic[16]. We demonstrated how coupled threshold oscillators (neurons) may be used to perform binary logic in a manner entirely

consistent with modern computer architectures.

Figure 2(a) demonstrates a viable circuit schematic for half-adder implementation using two neuronal oscillators labelled O_1 and O_2 and two inputs I_1 and I_2, which may themselves be the output from other neurons in a more complex circuit. In order to perform the logical operations it is necessary that either neurons with differing thresholds be used or the connections between the neurons should be of differing weights, indicated by w_1 and w_2 in the figure. Figure 2(b) displays a time series plot of a binary half-adder. Schematics and time series for a two oscillator full adder, a three oscillator seven input full adder, a 2x2 bit binary multiplier and a set reset flip-flop (used for memory) are also displayed[16].

CONCLUSIONS

Future work: Our current work is structured in five main steps:

1. Growing neurons on chips. 2. Design of a new model of threshold logic based on biological neurons. 3. Simulation of threshold oscillator logic using a suitable mathematical package. 4. Building neuronal logic circuits using neurons. 5. Growth of Alzheimer/vascular dementia diseased neurons on chip and measurement of neuronal degradation for different citicoline dosages.

This methodology will allow for the sub-cellular resolution (micro-pixel) within neurobiological preparations for example neuron-neuron interfaces and indeed later, entire neuronal networks. This proposed methodology has potential application in all areas of neuroscience, medical diagnostics and pharmacology.

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