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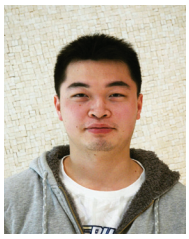
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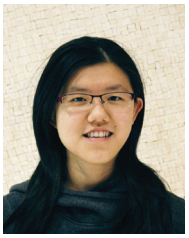
Lisa Zhao

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Letter from the Editors

SCIENCE, like so many worthwhile things in our lives, can seem like a bittersweet endeavor. Failed experiments, non-specific antibodies, and malfunctioning equipments abound, tempting so many of us involved in research to question why we do it at all. But there is something that keeps us engaged in research, despite its misgivings.

In this 8th volume of the University of Toronto Journal of Undergraduate Life Sciences (JULS), we draw on the experiences of Alan Lloyd Hodgkin and Andrew Huxley, both of whom received the Nobel Prize in Medicine and Physiology 50 years ago for their seminal work on action potentials. Hodgkin and Huxley began their work on electrical transmission along nerve fibers in frog sciatic nerves, which proved challenging given their small sizes. They ingenuously overcame this obstacle by using the giant axon of the Atlantic squid, whose large size made it conducive to recording ionic currents. Then came the problem of measuring the nerve impulses themselves, which only last fractions of a millisecond. Once again, they managed to overcome this technical hurdle by developing the voltage clamp, marking a technological breakthrough in electrophysiology.

Since then, we have witnessed spectacular conceptual and methodological advances in our understanding of the nervous system, from molecular and cellular biology to neuroimaging. Their discoveries helped launched a new frontier for investigation, bridging generations of scientists together in trying to understand the biological substrates of phenomena that have provoked centuries of philosophical and scientific inquiry. Certainly, we owe to Hodgkin and Huxley the wealth of knowledge their discoveries have sparked. But perhaps, we owe even more to the duo the affirmation that insights gleaned from overcoming ostensible failures is what makes scientific research so valuable.

In this issue, we commemorate the legacy of Hodgkin and Huxley by highlighting the work of undergraduate researchers who continue their tradition of resilience and ingenuity. From molecular aging to vaccines for RNA viruses, we are proud to present the increasingly impactful research being conducted by our peers in a wide array of disciplines. These are but a sampling of the intriguing and novel research that characterizes the undergraduate scientific community here at the U of T.

Of course, the publication of this issue would not have been possible without the dedication and expertise of our executive team and faculty advisors. We thank them for their invaluable insights and continued support. We would also like to thank the authors not only for their high quality submissions, but also for their perseverance and patience throughout the publication cycle.

Although it was not possible to showcase the entire breadth of undergraduate research at U of T in this single issue, we hope that you are nevertheless inspired to learn and engage in the research questions that resonate with you. Naturally, we also hope that you will consider contributing your work and talents to our future issues.

With best regards,

Lucy Ching Chau & Benedict Darren
Co-Editors-in-Chief, 2013-2014

Saturated Lewis Acids: A Potential Tool for Catalytically Managing Greenhouse Gases

Imindu Liyanage

In 1923, Gilbert Lewis published a landmark theory wherein he revolutionized the characterization of acid/base reactions by redefining them in terms of the exchange of electrons, rather than protons. He specifically identified two novel species: the Lewis Base, which donates an electron pair via a nucleophilic attack; and the Lewis Acid, which accepts this electron pair [1]. For much its history, it was assumed that these Lewis acids must be, by virtue of their chemical behaviour, unsaturated or lacking a full complement of electrons in its coordination sphere; for a full network would obviously be incapable of efficaciously accepting additional electrons. Indeed, most Lewis acid/base reactions do exhibit this trend; however recent work at the Stephan Lab has made significant strides at disproving this premise, where it now seems that a Lewis acid need not be unsaturated at all, and that saturated species can in fact be highly Lewis acidic[2].

From a theoretical perspective, a saturated Lewis acid is exceptionally problematic as most Lewis acids derive their reactivity from unsaturation[2]. This occurs as unsaturation translates to vacant electron orbitals; and it is these which can actually accept electrons during an acid/base reaction. For example, boranes (BH_3) are trigonal planar in shape and are potent Lewis Acids as they have a vacant p orbital running perpendicular to the plane of the hydrogens. Conversely, a saturated molecule such as a 4-coordinate phosphonium salt would appear to be a poor Lewis acid as its ligands already occupy the bonding orbitals – thus appearing to preclude further electron acceptance. It is noteworthy that the anti-bonding σ* orbital would be vacant in this species, however occupying this orbital under normal circumstances would require such a large investment of energy that the resultant molecule would be highly unstable, hence it does form. Indeed, most saturated species, including most of the aforementioned phosphonium salts are unreactive towards electron donors.

In a recent publication in Science – Prof. Stephan and colleagues have shown that by forming the salt with highly electrophilic substituents, the Lewis acidity of phosphonium can be greatly increased. This is achieved by using the ligands to draw electron density away for the phosphonium centre – making it decidedly more electron deficient; this deficiency increases the electron affinity of the phosphonium and this in turn can be harnessed to add electrons to the σ* orbital[2]. The precise molecule studied was an organofluorophosphonium (a phosphorus bonded to a fluorine and fluorinated aromatic ligands), and it was sufficiently Lewis acidic to successfully react with and form adducts when exposed to a Lewis base[2]. However, the organofluorophosphonium molecule displayed such potent Lewis acidity that it was also able to activate and cleave carbon–fluorine (C-F) bonds in fluoroalkanes (via a catalytic hydro-defluorination in the presence of silanes)[2]. This ability to manipulate a C-F bond has immense potential in the field of climate change as several notable greenhouse gases including chlorofluorocarbons, hydrofluorocarbons and perfluorocarbons all contain these bonds[3]. Therefore, given their extraordinary catalytic proclivities, saturated Lewis acids may prove to be the basis of an efficient long term solution for managing atmospheric concentrations of these greenhouse agents. In summary, this novel field of saturated Lewis acids promises to be an intriguing area of future study.

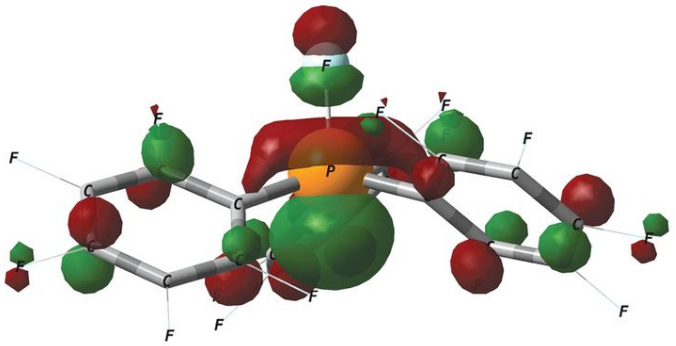

Figure 1 A space filing representation of the electrons in the remarkable fluorophosphonium salt [2]

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PPARs and Sex Hormones: Implications for the Gender Disparity in Multiple Sclerosis

Farzan Pavri and Charles Lee

Autoimmune disorders arise when the body falsely recognizes endogenous tissue as foreign. Often, the tissue provokes an immune-mediated response, ultimately resulting in a progressive and chronic disease course for the individual [1]. One such autoimmune disorder is Multiple Sclerosis (MS), whose disease course is characterized by degeneration of oligodendrocytes (the cells responsible for myelin formation around axons) in the central nervous system, leading to the hallmark, scar-like plaques [1].

As with most autoimmune disorders, a sexual dimorphism exists for both the incidence and pathophysiology of MS [2]. The gender disparity in MS incidence has steadily widened over the past 50 years, with a current ratio around 3:1 (female: male) [2].

The exact etiology of MS remains unknown; however, in light of the epidemiological data, an intuitive starting point for elucidating the disease mechanism would be to explore the reasons behind the gender disparity. Such is the approach taken by Dr. Shannon Dunn, Assistant Professor within the Department of Immunology at the University of Toronto and Principal Investigator of the Dunn Laboratory.

In 2007, Dunn *et al.* proposed a pathway that may contribute to the sex bias in MS. The study examined the interactions of a protein known as Peroxisome Proliferator–Activated Receptor alpha (PPARα), part of the nuclear receptor superfamily, with molecules involved in immune response [3]. In T cell populations, PPARα reduces Th1-mediated immune responses [3]. In a mice model, PPARα was observed to be differentially expressed between male and female mice, with males exhibiting increased expression in T cell populations [3]. This is consistent with the finding that PPARα expression is positively correlated with the levels of circulating androgens [3].

To substantiatte the preliminary findings of Dunn *et al.* (2007), Angela Zhang, a Ph.D. candidate in the Dunn Laboratory, investigated whether a sex bias exists in the regulatory role of PPARα and PPARγ in cytokine production.

In a recent study, Zhang *et al.* (2012) demonstrated that the CD4+ cells from women are more robust, proliferate more, and produce higher levels of IFNγ than the cells from men in response to the same antigens. All these factors contribute to the stronger autoimmune response in women, and may explain the sexual dimorphism in the incidence of MS [4]. Additionally, through the use of PPAR-specific siRNAs, the study showed that knockdown of PPARα increased IFNγ levels in males whereas knockdown of PPARγ increased IL-17A levels in females [4].

To account for the sex difference in CD4+ T helper cells, the study also examined the potential role of the androgen Dihydrotestosterone (DHT) and discovered that DHT is associated with the increased expression of PPARα but the decreased synthesis of IFNγ [4]. The results suggest that androgens may play an important role in lowering autoimmune response, by indirectly reducing cytokine production [4].

The elucidation of PPARs and their interactions with hormones provides insight into both MS therapy and disease mechanism. The association amongst PPARs, hormones, and autoimmunity opens the door for understanding many potential mechanisms responsible for MS sexual dimorphism. For instance, differential expression of PPARs (mediated by androgen levels) may contribute to the increased prevalence in females [3]. Personalized medicine is one of the many frontiers of translational research, and the sexually dimorphic nature of MS may make it an excellent candidate for gender-based treatments [4].

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Retinal stem cells: A possible therapy for Age-related Macular Degeneration?

Jasmina Uzunovic

Age-related macular degeneration (AMD) is the most common form of blindness for those over the age of 55, affecting millions around the world. Blindness is a result of degeneration of the retinal pigmented epithelium (RPE), the layer of cells at the back of the eye that absorbs light and nourishes the photoreceptor cells. Without nourishment photoreceptor cells, rods and cones perish, resulting in loss of vision. Despite the known etiology of AMD, there is currently no way to regenerate these lost cells and restore vision.

Regenerative medicine is a promising approach with the potential of restoring vision to those afflicted with degenerative blindness. A few clinical trials have already been conducted where transplanted stem cells led to a renewal of sight. These stem cells were isolated from aborted fetal eyes, differentiated into RPE, and successfully transplanted into patients without immune rejection. While promising, this avenue of treatment still faces many problems. Ethical concerns regarding the fetal source of the stem cells in these trials prevent this form of therapy from having widespread applications. Moreover, around 6 years after receiving the transplant, patients began to lose sight once more, indicating that this treatment is only a palliative remedy, rather than a permanent solution [1].

Derek van der Kooy's, a professor in the Molecular Genetics department at the University of Toronto, was the first to discover adult retinal stem cells (RSCs) in the mammalian eye, first in mice and subsequently in humans [2, 3]. These stem cells are located in the pigmented ciliary margin in the periphery of the eye. The van der Kooy lab has also been able to direct the differentiation of RSCs into rod photoreceptors with an efficiency exceeding 90%, making them a promising source of rods to replace those damaged or lost due to disease [4].

It is well established that many lower vertebrates, such as zebrafish and teleosts, have the ability to regenerate neuronal cells in their eyes after injury through the proliferation, migration and differentiation of adult RSCs. Essentially, these animals can repair their own retinas. It is hypothesized that mammalian RSCs are evolutionarily homologous to these other vertebrates, but are kept quiescent through inhibitory factors present in the mammalian eye [5]. The van der Kooy lab has worked to characterize the origins of these inhibitory signals. RSCs are kept inactive by the nearby cornea, which secretes bone morphogenic protein 2 (BMP) and BMP4, and the lens, which releases secreted Frizzled-related protein (sFRP) [5]. By determining how to block these inhibitory proteins, it may be possible to activate the proliferation of RSCs *in vivo*, which could then replenish the RPE and photoreceptors without any need for an invasive and traumatic transplant. Such a therapy would be both safer and produce longer term results than a transplant.

It is pioneering stem cell based regenerative research, like that carried out in the van der Kooy lab, that could lead to the University of Toronto playing an important role in developing therapies that could help patients suffering vision loss to see the light at the end of the tunnel.

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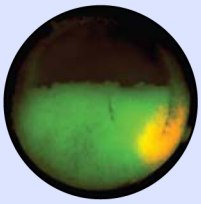

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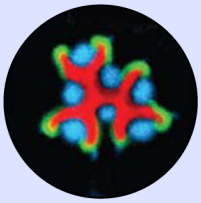

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JULS RESEARCH

Distribution of Cholecystokinin Containing Neurons in the Amygdala of Mice

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Abstract

The amygdala regulates fear learning and anxiety but the critical internal connections between amygdalar subnuclei are poorly understood. Cholecystokinin (CCK) is found within a subfamily of GABAergic neurons which modulates the generation of anxiety in the amygdala. In this experiment, expression levels of the endogenous CCK8S peptide, a biologically active isoform of cholecystokinin, in amygdalar subnuclei of C57BL/6 mice were studied using immunohistochemistry. Utilizing this method, we were able to observe the expression levels of CCK in the amygdala and propose a possible anatomical connection between its subnuclei. In this experiment, we detected large populations of GABAergic CCK-containing neural cell bodies in the basolateral amygdala (BLA) with some expression in CCK terminals on non-GABAergic cells. In the central nucleus, strong CCK expression was seen in axon terminals. Furthermore, the main intercalated nucleus (IM) of the amygdala showed connections between dopaminergic terminals and CCK8S positive neurons. This suggests that dopaminergic neurons project to the CCK-containing cells. Finally, many GABAergic CCK-containing neurons in the medial nucleus (MeA) were observed. Therefore, expression of endogenous CCK in the BLA, CeA, IM and MeA at a cellular level was detected. According to these expression levels we proposed that in response to anxiety, CCK should inhibit excitatory neurons in the BLA and inhibitory GABAergic neurons in the CeA. This will cause a net excitation in the CeA which further leads to an excitatory response in brainstem nuclei and induces anxiety related behavior such as startle. IM and MeA however are regulatory nuclei which project to the BLA.

Introduction

Extensive evidence indicates that the amygdala is important for fear learning, conditioned fear and the generation of anxiety [1-4]. Microcircuitry of the amygdala in conditioned fear response has been well studied but the mechanism underlying unconditioned fear and anxiety at a cellular level requires greater investigation [5,6]. The amygdala is composed of multiple sub-nuclei each with unique morphology and neurons that are highly interconnected [7]. The lateral nucleus of the amygdala receives inputs from the sensory system and projects to the central, basal and intercalated sub-nuclei [1]. Output signaling from the basal nucleus (BN) to the ventral striatum initiates avoidance or freezing whereas projections from the central nucleus (CeA) to the brainstem lead to arousal and escape [2]. Approximately 90% of the neurons in the basolateral amygdala (BLA) are glutamatergic many of which project to CeA [8]. The CeA is divided into the lateral (CeL) and medial (CeM) subdivisions. CeL is strongly GABAergic and projects to CeM. CeM sends axonal projections to the brainstem CeL receives excitatory inputs mainly from the BLA and inhibits the excitatory cells in the CeM [9,10]. Feed forward inhibition form CeM further inhibits the brainstem nuclei responsible for fear response. This suggests that the excitatory neurons in the BLA, under normal circumstances are anxiolytic [5]. If the BLA is inhibited, anxiety is generated through activation of the CeM excitatory neurons [11]. A small subpopulation of GABAergic neurons which may inhibit BLA glutamatergic cells are cholecystokinin (CCK) containing GABA neurons that are well known for regulation of anxiety [12,13].

Cholecystokinin (CCK) is derived from a 115 residue prepro-CCK peptide which is cleaved into smaller, biologically active isoforms such as CCK4, CCK8 and the most abundantly found, CCK8S [12,14,15]. CCK is expressed in the cell bodies and terminals of many neurons including dopaminergic, serotonergic, opiate producing, glutamateergic and GABAergic cells with modulatory actions [15]. The biologically active components of cholecystokinin bind to CCKA and CCKB receptors where CCKB is expressed more abundantly in the brain, particularly in the limbic system [16]. Cholecystokinin binds to its CCKB receptor and induces anxiety and even panic at higher doses, while a decrease in CCK activity can have anxiolytic effects [14-20]. In the amygdala, CCK receptors are found mainly on GABA interneurons of the lateral (LA) and baso-lateral (BLA) subnuclei [11]. The peptide is produced by a subpopulation of GABA interneurons in the BLA which primarily express CCKB receptors [21]. Immunoreactive studies however, propose that not only the BLA but CeA and IM contain CCK [15]. Electrophysiological studies in amygdala slices indicate that activation of CCKB receptors, enhance potassium-dependent release of GABA and induction of inhibitory post synaptic potentials (IPSPs) on the glutamatergic neurons of the BLA [11,15]. Therefore BLA-CeL-CeM circuitry is necessary for regulation of anxiety [21].

Other studies have shown that the intercalated nuclei (ITC) and the paracapsular islands of the amygdala are also important for the regulation of fear and anxiety [22]. These nuclei are clustered around the basolateral complex where they receive inputs from the BLA and the cortex and project back to the BLA, CeA and MeA, regulating the main inputs and outputs of the amygdala [22,23]. Intercalated cells (ITCs) are dominantly GABAergic with dopaminergic terminals. ITCs provide feed forward inhibition to the BLA and CeA neurons which can induce anxiety [22,23,24]. The MeA of the amygdala or the extended amygdala receives strong input from the olfactory system [25]. Receptors for gonadal hormones are also widely expressed in this nucleus suggesting that the MeA is important for regulation of sexual and social behaviors and not generation of anxiety [25,26,27]. However, it is proposed that exposure to predator odors or activity of estrogen receptors in the MeA can induce anxiety in mice [28]. Little evidence of the presence of GABAergic CCK containing cells in the MeA and their interactions between MeA and BLA - CeA were found previously [29]. In our anatomical study, expression of CCK8S in the BLA, CeA, IM and MeA of the amygdala in C57BL/6 mice using fluorescence imaging was examined. This allows better understanding on how the endogenous CCK may cause a net excitation or inhibition in amygdalar subnuclei and how these nuclei are interconnected. We expected our results to be consistent with the known internal circuitry of the amygdala and to observe high expression of CCK in each of these subnuclei. This expression however, should vary from one nucleus to another.

Materials and Methods

Mice

C57BL/6 mice (5-8 months old) were used. All 20 mice were healthy and sacrificed under anesthesia using perfusion technique [30]. Their brains were then extracted, kept in 4% paraformaldehyde (PFA) for 24 hours and were fixed in hypertonic sucrose solution for another 24 hours. Brains were then sliced using a Leica1850 cryostat.

Immunohistochemistry

Primary antibodies used in this experiment were rabbit anti-CCK8S (1:800) provided by Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA), rabbit anti-tyrosine hydroxylase (TH) (1:800) and mouse anti-GAD65 (1:500), shortly referred to as GAD, for dopaminergic and GABAergic neurons respectively were provided by Abcam (Abcam, Cambridge, MA, USA). All antibodies were diluted in 7.4pH PBS (NaCl 137mM, KCl 2.7mM, Na¬2HOP4 10mM, KH2PO4, 1.8mM) and all slices were treated with 16% normal goat serum prior to staining. Incubation period for each antibody was: CCK8, 96hrs at 4°C, TH, 24hrs at 4°C and 1hr at room temperature (20°C) and GAD65, 48hrs at room temperature. Secondary antibodies used were goat anti-rabbit (Alexa Fluor 488) or goat anti-mouse (Alexa Fluor 596), both provided by Abcam. Incubation period for secondary antibodies was 90 minutes at room temperature. Cell nuclei were stained with DAPI. Sections were examined using an FSX100 Olympus microscope equipped with standard sets for blue light excitation. Images were analysed using cellSense Viewer software. Sections treated only with secondary antibody were used as control in order to detect any possible unspecific protein binding.

Detection of colocalization

In order to detect if the expression of an antibody was mainly intracellular or extracellular, we used anatomical techniques in which if the antibody had a strong expression and surrounded the cell nucleus as a large cluster, it was concluded that the expression was in the soma. If the expression was stronger but did not surround the nucleus and was expressed as distinguishable dots with 2-3μm in size, it was assumed that the expression was mainly on axon terminals or dendrites. This suggests that neurotransmitters are released from pre-synaptic neuron or binding to their receptors on the post-synaptic cell in the synaptic cleft and hence, located outside of the neuron. Cell counts were done manually in different brain slices for each desired nucleus. Numbers of mice used to obtain colocalization data were 5, 5 and 4 for BLA/CeA, MeA and IM respectively and 5 were used as control.

Statistical analysis

In this experiment, numbers of cells expressing a desired colocalization and the total numbers of cells present in each viewed section under the microscope were counted manually. These numbers were then compared among all mice and Standard deviation was computed for significance test calculations. These data from all sections were then averaged and converted to percentage values.

Results

Variable distributions of GABA, dopamine, and CCK8s were found within the BLA, CeA, IM and MeA. GAD65 positive neurons were widely expressed in the amygdala. In the BLA, strong colocalization between CCK8S and GAD65 in the neural soma was observed in 25% of the BLA cells (Figures 1-2). CCK containing-neurons are a subpopulation of GABAergic neurons and CCK mRNA is widely distributed across the BLA [16]. Therefore, colocalization was expected. Approximately 10% of BLA GAD-positive cells did not show any expression of CCK. 60% of the neurons in the BLA were not GAD-positive but expressed CCK on their axonal terminals and 5% of cells expressed neither GAD nor CCK. In the CeA, CCK expression was much stronger than the BLA which was predominantly extracellular. 90% of CeA neurons were GAD-positive and their somas were surrounded by CCK. This suggests that these cells receive strong inputs from CCK containing axons. 3% of the neurons in this region were GAD positive with intracellular CCK expression.

The remaining neurons were GAD negative and CCK expression was detected on their terminals only (Figure 3). Intercalated nuclei and paracapsular islands of the amygdala are also important for regulation of fear and anxiety [22]. These nuclei contain large populations of GABAergic cells which receive dopaminergic inputs [23,24]. Staining for tyrosine hydroxylase (TH) allowed the detection of the IM at a macroscopic level. CCK8S detection under lower magnifications however, was difficult due to little CCK expression in this region (Figure 4). It was evident that TH had strong extracellular expression on 50% of the IM neurons. This expression was on axon terminals suggesting that at least half of IM neurons receive dopaminergic inputs. CCK expression in this region was predominantly intracellular and limited to 20% of neurons. Approximately 75% of CCK positive cells expressed TH on their terminals. In addition, a total of 24% of neurons were TH and CCK negative. We were not able to detect paracapsular islands or other intercalated nuclei (Figure 5).

In the medial nucleus, clear intracellular colocalization between GAD65 and CCK8S was found. Less than half (45%) of the neurons in MeA were GAD positive and approximately 35% of them expressed CCK. CCK expression was only limited to GAD positive neurons (Figures 6-7).

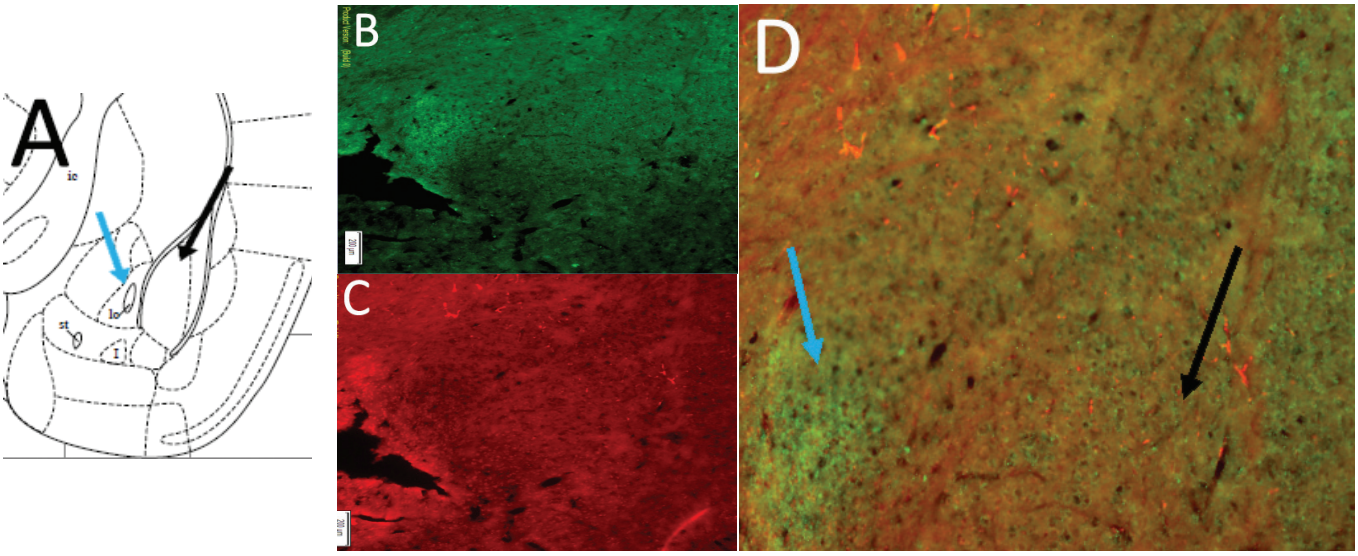

Figure1. (A) Relative location of the amygdala and its subnuclei in the mice forebrain. The black arrow is pointing at the basolateral amygdala (BLA) and the blue arrow is pointing at the central nucleus (CeA). (B-C) Expression of single labeled antibody in the BLA and CeA green shows CCK8S peptide and red represents GAD65 for GABAergic neurons. (D) Colocalization between CCK8S (green) and GAD65 (red) in the BLA and CeA. High quantity of CCK containing GABAergic neurons were observed in the BLA but overall, the expression of CCK was more apparent in the CeA. No colocalization between CCK8S peptide and GAD65 was seen at the soma level in the CeA under lower magnifications but Instead, strong CCK expression was observed around the cell bodies of GAD65 positive neurons in this region.

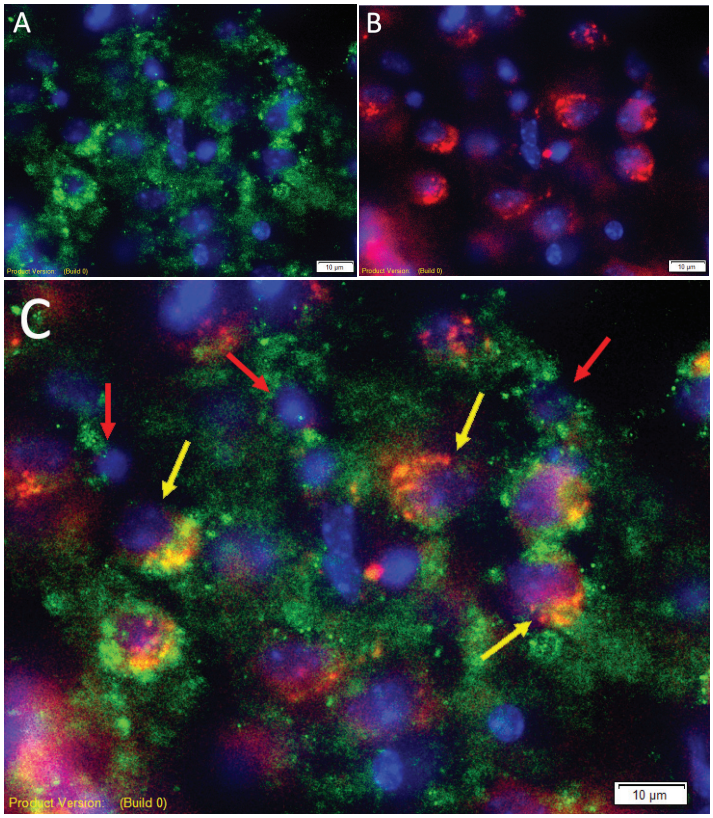

Figure 2: Expression of CCK8S (green) and GAD65 (red) in the basolateral amygdala (BLA). (A-B) Single labeled expression of CCK8S and GAD65 in GABAergic neurons.(C) Colocalization of CCK8S/GAD65containing GABAergic neurons (indicated by yellow arrows). 23% of cells expressed both GAD and CCK in their cell bodies suggesting that the peptide is synthesized in these GABAergic neurons. 60% of BLA cells were not GAD positive but received inputs from CCK containing axons pointed to by red arrows. Cellular nuclei are stained blue with DAPI.

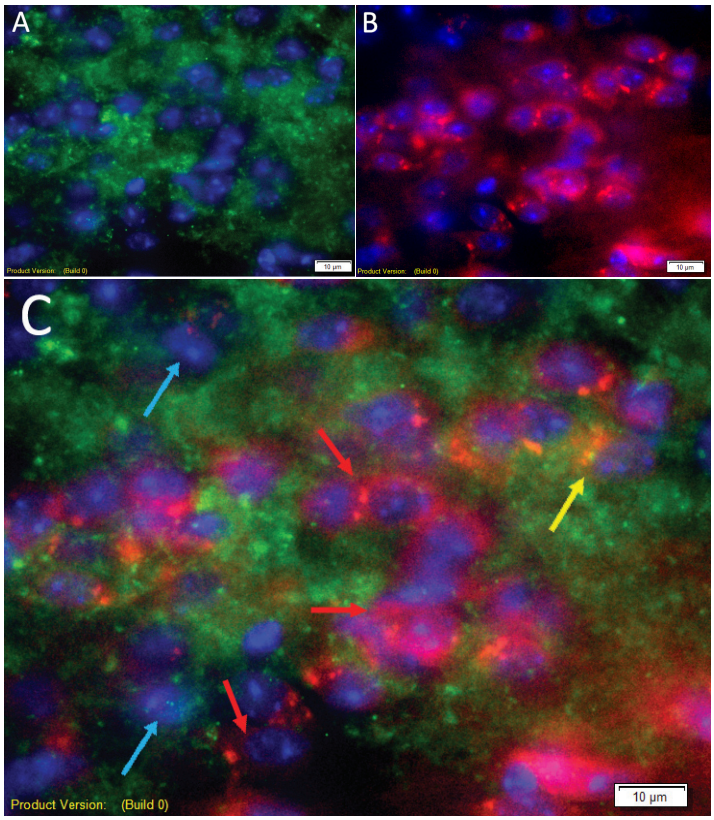

Figure 3: CCK8S (green) and GAD65 (red) expression in the central nucleus of the amygdala (CeA). (A-B) Single labeled CCK8S and GAD65 in CeA. (C) Colocalization between CCK8S and GAD65. Very little colocalization between GAD65-positive neurons and CCK8S peptide was seen at cell body level (indicated by yellow arrow). 90% of GAD positive CeA cells showed CCK expression on axon terminals that project to the soma of these GAD positive neurons (pointed to by red arrows). The rest of the cells (5%) were GAD negative and had no expression of intracellular CCK (pointed to by blue arrows). Cell nuclei are stained blue with DAPI.

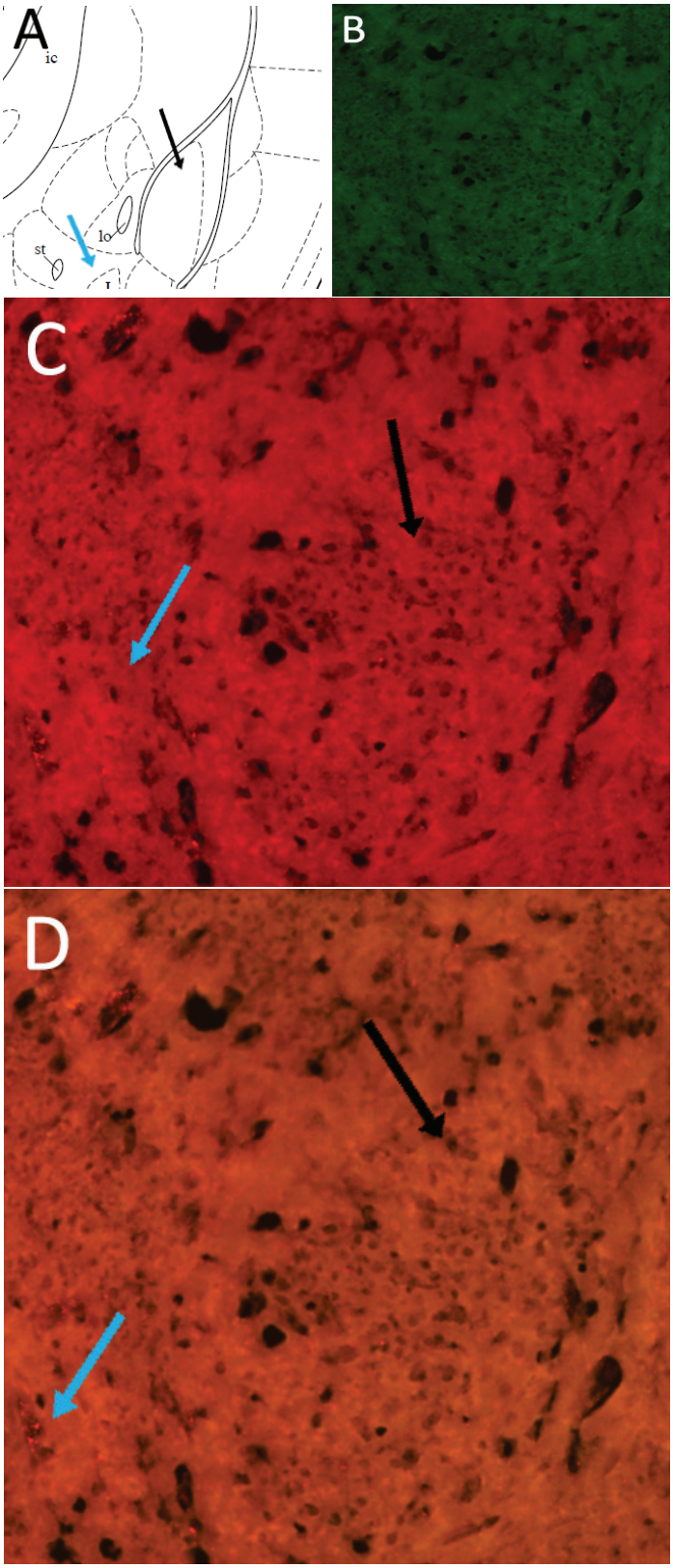

Figure 4: (A) Relative location of the amygdala and its subnuclei in mice forebrain. The black arrow is pointing at the basolateral amygdala (BLA) and the blue arrow is pointing at the main intercalated nucleus (IM) (C, D). Expression of single labeled antibody in the BLA and IM where green shows CCK8S peptide and red represents TH. (D) Colocalization between CCK8S and TH in the BLA and IM. Strong expression of TH containing cell was nto seen in the BLA 50 % of the IM cells were TH positive. CCK8S expression however was detected in only 20% of the IM cells. No clear colocalization between CCK8S and TH at a macroscopic level was observed.

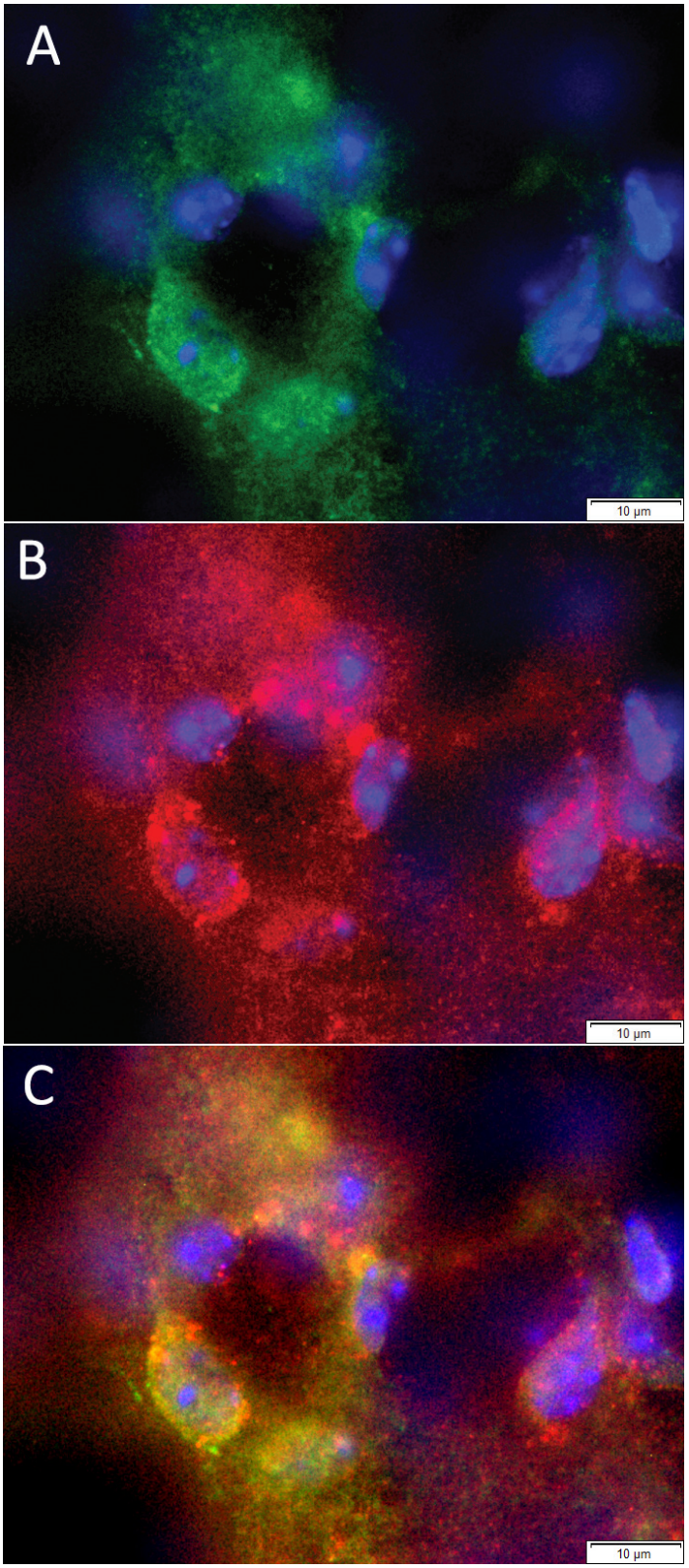

Figure 5: Expression of CCK8S (green) and Tyrosine hydroxylase (red) in the IM. (A-B) Singly labeled expression of CCK8S and TH in IM. (C) Colocalization between CCK8S and TH. CCK is strongly expressed in the soma but TH expression is mainly extracellular, on axonal terminals that project to such CCK-containing neurons. Half of IM neurons receive dopaminergic inputs where 75% of these cells are CCK-positive. Cell nuclei are stained blue with DAPI.

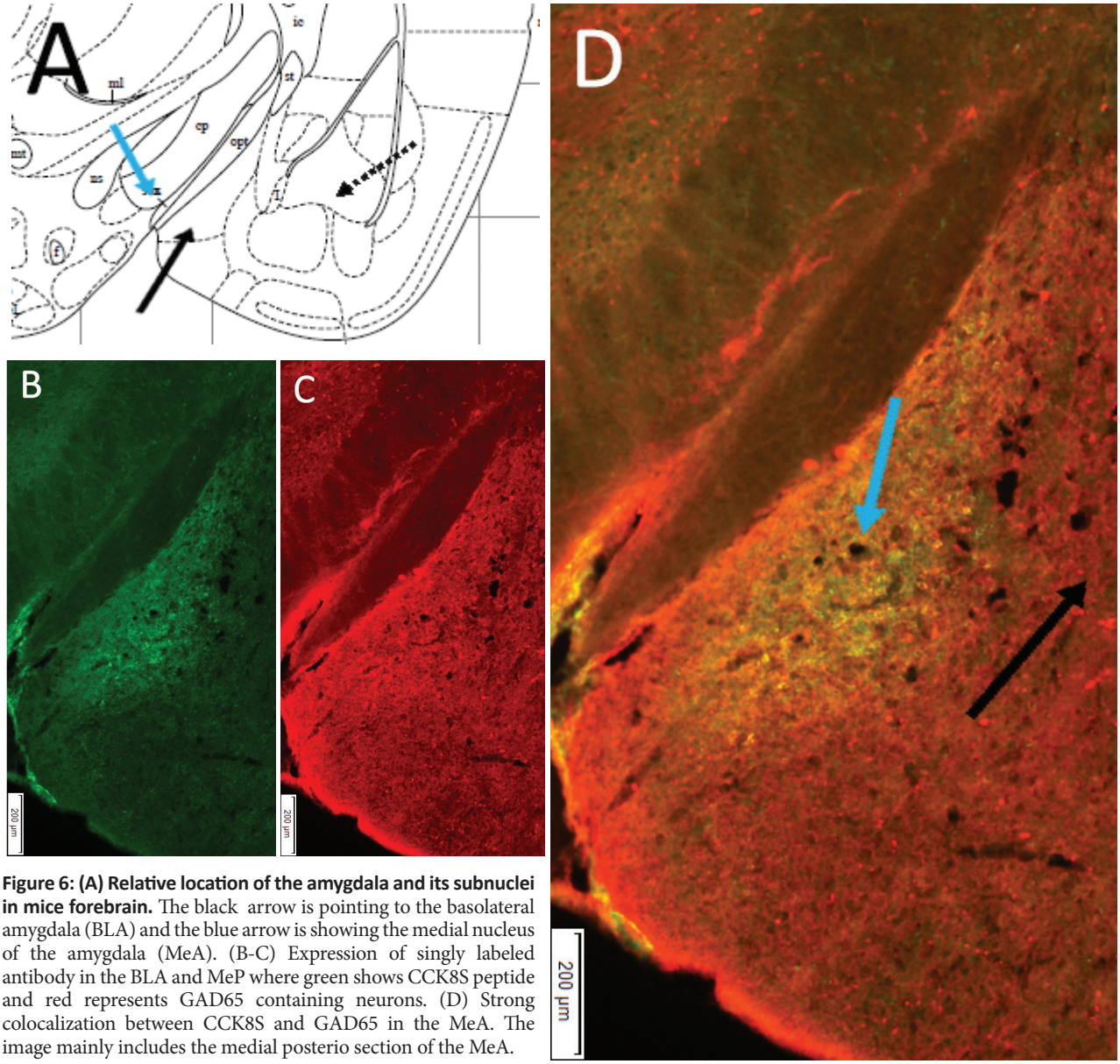

Figure 6: (A) Relative location of the amygdala and its subnuclei in mice forebrain. The black arrow is pointing to the basolateral amygdala (BLA) and the blue arrow is showing the medial nucleus of the amygdala (MeA). (B-C) Expression of singly labeled antibody in the BLA and MeP where green shows CCK8S peptide and red represents GAD65 containing neurons. (D) Strong colocalization between CCK8S and GAD65 in the MeA. The image mainly includes the medial posterio section of the MeA.

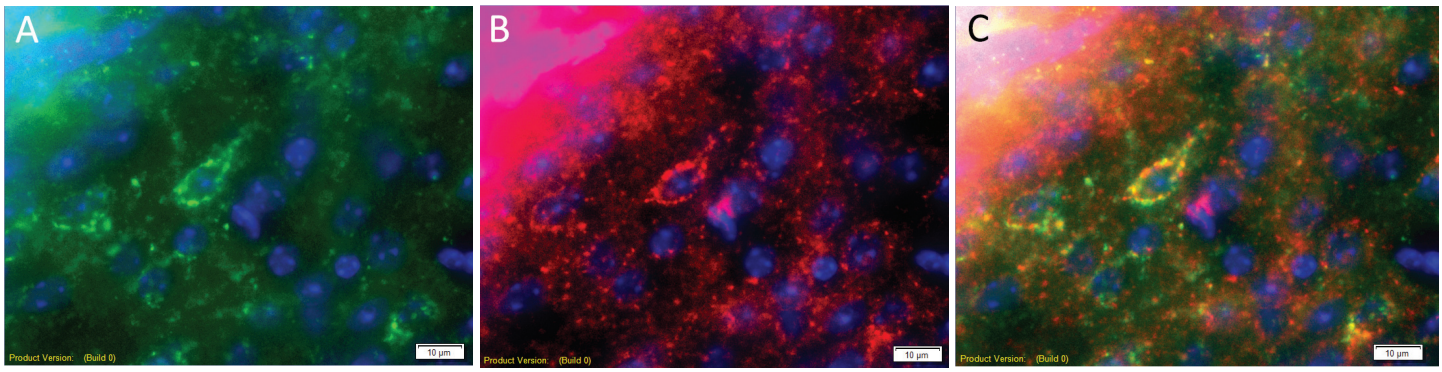

Figure 7: Expression of CCK8S (green) and GAD65 (red) in the medial posetrio-dorsal nucleus of the amygdala (MePD). (A-B) Singly labeled expression of CCK8S and GAD65 in MePD. (C) Colocalization between CCK8S and GAD65 was predominantly intracellular. 45% of all MePD neurons are GAD positive and 35% of them express CCK. CCK expression, however, is only limited to GAD positive cells. Cell nuclei are stained blue with DAPI.

Conclusion and Discussion

Cholecystokinin is an anxiogenic substance and inhibition of CCK activity is anxyolitic [17,19,32,33,34]. These conclusions were made according to the activity of exogenous CCK. Anxiogenic properties or the anatomical localization of endogenous CCK peptide, however, are not completely understood. The purpose of this experiment was to improve our understanding of the internal circuitry of the amygdala based on the expression of the endogenous CCK by characterizing the localization of an active isoform, CCK8S. Strong CCK expression is seen in the BLA [35] and we demonstrated that CCK has clear intracellular colocalization with GABAergic neurons in this region. This suggests that perhaps CCK peptide is synthesized in these cells. CCK is also expressed on the terminals of non-GABAergic neurons in the BLA. It is possible to assume that these cells are excitatory glutamatergic neurons with CCK innervations since 90% of the BLA neurons are glutamatergic [8,12]. It is hypothesized that CCK is synthesized in the BLA and is release on CeA neurons, where they may or may not interact with CCKB receptors. Activation of CCKB receptors on glutamate neurons lead to an increase in potassium dependent release of GABA and induction of IPSPs on glutamatergic neurons in the BLA [11,15]. Inhibition of BLA excitatory neurons can in fact induce anxiety which may be regulated by innervations of CCK on glutamatergic cells. Some BLA cells were GABAergic but did not express CCK. These cells perhaps belong to other groups of GABA neurons such as parvalbumin (PV) or somatostatin (SST) subpopulations.

The central nucleus had the strongest CCK expression compared to other amygdalar subnuclei. CeA GABAergic neurons were surrounded by CCK peptide where it can perhaps bind to CCKB receptors, located on the surface of GAD positive cells. These receptors are excitatory G-protein coupled receptors [16]. Excitation of inhibitory GAD positive CeA neurons through the activity of CCKB receptors should enhance the feed forward inhibition on CeM neurons and induce an anxyolytic effect. It has been shown, however, that disinhibition of inhibitory CeL cells excites CeM glutamatergic neurons and elicits anxiety and freezing [5, 36]. Furthermore, expression of CCKB receptors on GABAergic CeL neurons is weak [37, 38]. Strong CCK expression on CeA terminals cannot be easily explained. It can be argued, however, that the CCK released from the terminals of CCK-GABAergic neurons in the CeA, diffuse long distances in the extra-cellular fluid through volume transmission to reach their target receptors (CCKB) on the BLA [15, 39]. Greater investigation is required to explain this observation.

Neurons in the main intercalated nucleus (IM) of the amygdala expressed CCK at the soma level. The intercalated nuclei (ITC) project to both BLA and CeA and receive strong innervations from the dopaminergic neurons. Some dopamine innervations on GABAergic - Parvalbumin neurons in the BLA previously found [27]. In the IM, dopaminergic inputs were detected on the terminals of inhibitory GABAergic neurons [27, 40]. Perhaps dopamine has a modulatory role on the activity of CCK-containing cells [31]. Activity of dopamine D1 receptor excites the inhibitory pathway and suppresses feed-forward inhibition to the basolateral and central amygdaloid nuclei [22]. Intracellular CCK expression in these cells suggests that perhaps dopaminergic inputs excite CCK containing cells and activation of these neurons can suppress BLA feed-forward inhibition. Presumably, in resting conditions, the main intercalated nucleus contributes to maintaining a low level of anxiety by generating feed-forward inhibition within the BLA and CeA. Projections of dopamine neurons, mainly from the ventral tegmental area [41, 42], to CCK neurons may lead to anxyogenic behavior. The medial nucleus of the amygdala (MeA) has strong expression of CCK in the soma of its GABAergic neurons that may have anxiogenic effects. Brief exposure to predator odor can induce anxiety in mice by alteration of mesolimbic enkephalin levels [28]. Induction of anxiety by predator scent may be regulated through CCK- GAD positive neurons in MeA but more investigation is required to prove this claim.

In our experiment, we were able to show the distribution of CCK in the amygdala in both GABAergic and non-GABAergic cells. Use of transgenic CCK-Cre line of mice can provide better expression of stained CCK, thus, better anatomical data although the Cre-lines themselves have certain limitations [44,45]. Staining for CCKB receptors may lead to a better understanding for how CCK interacts with its receptors in the amygdala. Finally, behavioral testing is important to see if CCK expression induces anxiety in all subnuclei of the amygdala. This can be achieved by micro-injection of this peptide to each sub-nucleus and test anxiety levels in the animal through startle or any other mechanism [19, 33]. It is important to note that multiple neurotransmitters or neuropeptides such as endogenous opioid system, Norepinephrine, dopamine and serotonin are also anxiety regulators and therefore may influence fear responses [46-49]. Thus CCK should be considered the most important as an anxiogenic substance.

Acknowledgements

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JULS RESEARCH

Molecular Dynamics Study of Radical-Induced Epimeric LSEAL Peptides

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Abstract

Calpain, a cysteine protease, has recently gained attention for its role in apoptosis induction, which could lead to neurodegenerative disorders such as Alzheimer and Parkinson diseases following the ischemic episode caused by stroke or cardiac arrest. The LSEAL (H-Leu-Ser-Glu-Ala-Leu-OH) pentapeptide is a synthetic amino acid sequence that has been found to be an effective calpain inhibitor. In the present study, radical-induced epimerization events in which the chirality of each alpha-carbon in the LSEAL pentapeptide is switched from L to D-configurations were independently simulated in context of complete peptide. The structural consequences of each free radical epimerization event were then studied using molecular dynamics (MD) methods. It was found that there are structural consequences as a result of epimerization, which may cause changes in the binding affinity of LSEAL to calpain. Hence, the study may be able to help in understanding the process of molecular aging, as epimerization of amino acid residues has been used as a measurement of molecular aging.

Introduction

Calpain, a calcium-dependent cysteine protease, is involved in cytoskeletal disruption and cell death as observed in the ischemic cascade [1]. The cascade, comprised of a series of reactions caused by the restriction of blood supply, results in localized cell death in aerobic tissue. Such restriction upsets bodily functions, particularly in the brain, and is characteristic of stroke and cardiac arrest [2]. The anaerobic respiration that is then necessary for cell survival triggers an increase in intracellular Ca^{2+} level that hyperactivates calpain, inducing apoptosis. Hyperactivated calpain is involved in various neurodegenerative disorders such as Alzheimer disease and Parkinson disease [3]. Thus, the search for calpain inhibitors has become increasingly important because of their use in delaying the apoptotic process, allowing treatment of the vessel blockage and impeding development of further apoptotic events [4]. In 2005, a pentapeptide sequence with such a function was discovered through phage display and selection by Guttmann and colleagues [5]. It contained the sequence NH_2-Leu-Ser-Glu-Ala-Leu-Ac (LSEAL) (Figure 1) and decreased the protease activity of the calpain complex [4]. Experiments using Western blot demonstrated that the addition of LSEAL to a calpain mixture reduces the enzyme activity by 50% [1].

The sequence and function of the synthetic peptide LSEAL are similar to those of calpastatin, a natural calpain inhibitor [6]. The binding of calpastatin and LSEAL to the calpain enzyme is reversible, and the calpain-calpastatin balance plays a vital role in the maintenance of physiological homeostasis [7]. This interaction can be manipulated to regulate calpain activity, making LSEAL a plausible candidate for future research in the treatment of symptoms following ischemia [3].

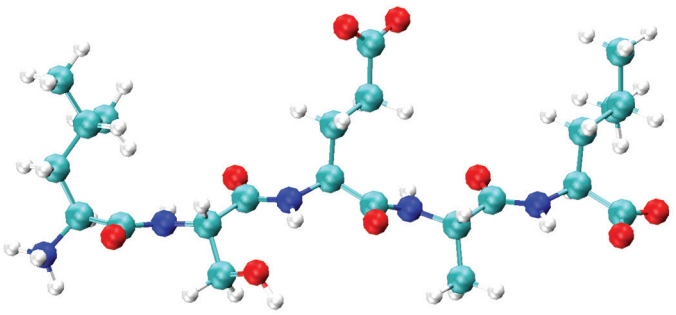

Figure 1: Molecular structure of LSEAL pentapeptide showing leucine, serine, glutamic acid, alanine, and leucine residues in order.

The LSEAL sequence was found to be conserved within several long peptides that have the ability to bind to calpain's active site and inhibit its enzymatic activity [4]. Recent research allows for a reasonable assumption that LSEAL is unstructured or only partially folded until it is bound to the target protein, at which point it will undergo a conformational change to fit the protein active site. LSEAL may exhibit two possible conformations – the twisted and horseshoe forms (Figure 2) – upon binding to the calmodulin-like domain (CaMLD) of calpain [7]. The twisted form, which resembles an alpha-helix, is more abundant in cytoplasm than the horseshoe

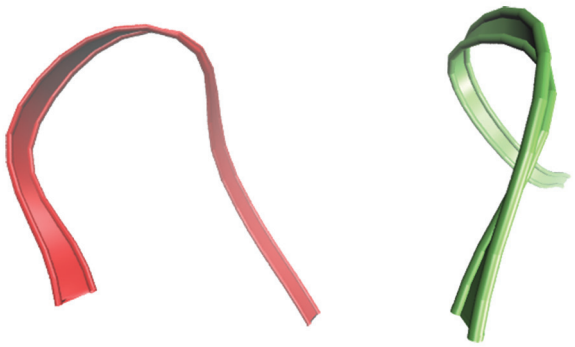

Figure 2: Horseshoe (red) and twisted (green) conformation of LSEAL. While both are considered native conformations of the peptide, the twisted conformation is more abundant in the cytoplasm whereas the horseshoe conformation is more abundant at the calpain active site.

form. However, the horseshoe form has a tendency to transform into the twisted form due to the amphiphilic side chain's ability to adapt to the polar environment. Calculations based on hybrid distance geometry-dynamical simulated annealing method using each of the forms as a starting point show that both conformers are considerably stable [1]. Therefore, both conformers are concluded to be native conformations [7].

LSEAL's binding location on calpain and its binding structure were determined via High Ambiguity Driven Docking protocol and X-ray spectroscopy [2]. Unexpectedly, the twisted form is found to have a lower occupational frequency at calpain's active site than the horseshoe form but both conformations are found at equal probability. This is because the secondary structure of the horseshoe form is better at superimposing onto CaMLD, while the twisted conformer has a smaller structure and often slides through the binding site, forfeiting its advantage in having higher abundance [7].

While LSEAL aims to mitigate calpain's action in inducing apoptosis, this process can be complicated by the other ongoing reactions in the body. One of such is the oxidative stress posed by overabundant reactive oxygen species (ROS), which could lead to changes in chirality of the backbone carbon atoms in the LSEAL peptide. ROS, present in most biological systems, are a part of normal cellular metabolism by the mitochondria and are sometimes involved in signaling pathways. However, they are also capable of imposing oxidative stress on the cells [5]. ROS include radicals and reactive molecules, examples are singlet oxygen, superoxide, hydrogen peroxide, and hydroxyl [3,4]. Targets of these nucleophilic molecules include proteins, lipids, and nucleic acids [6]. One of the physiological consequences of oxidative stress is aging – a process by which a cell's ability to proliferate is decreased as protein components are broken down or otherwise rendered dysfunctional by ROS [7].

As individuals grow older, ROS accumulate in the body and gradually impair the functionality of existing proteins, especially in individuals with chronic inflammation [8]. It is important to note that the human body only synthesizes proteins using amino acids in L-configuration [9]. Under oxidative stress, an amino acid residue may be attacked by the oxidative species at the hydrogen–Cα bond. The oxide radical usually abstracts a proton from the Cα, leaving a carbon radical at the alpha position [10]. The Cα-centered radical can then accept another hydrogen atom from a proton donor such as H_2O_2, forming a new residue which has an equal probability of adopting either L or D-configurations [10]. The transformation from the L to D-configuration is an epimerization process which produces a stereoisomer with opposite chirality from the original. The chirality change will impact the conformation of the molecule and most likely cause destruction to the peptide function [11]. Other oxidative reactions with proteins may involve polypeptide backbone cleavages, protein-carbonyl derivative generations, or protein-protein cross-linkages [12].

The focus of this paper is to examine the LSEAL pentapeptide in its native configuration with all amino acid residues in L-configurations, and to compare the structural differences between LSEAL and its five epimers which contain D-configured residues at various positions within the peptide. The most energetically stable conformations are obtained from computational models to inspect the properties of each epimer.

The computation was conducted *in silico*, giving the simulative experiments a list of advantages. Rigorous mathematical boundaries were applied in a quantum mechanical setting with an agreeable degree of accuracy, and no real-life experimental materials are required. This approach is helpful for investigations into relatively new topics, in that experimental errors can be easily fixed at a minimal cost.

Materials and Methods

The linear structure of the pentapeptide LSEAL was created using the *tleap* module of AmberTools 12 software suite [13]. In addition to the wild-type structure, five peptides were constructed, each with the alpha-C of one of the five residues in the D-configuration. This was done by inversing the z-axis coordinate of atoms pertaining to desired residue conformation. The program Visual Molecular Dynamics (VMD) was used to confirm the L → D-configuration change [14].

The Amber99SB force field was used during the conformational search with implicit solvent model [15, 16]. After initial minimization (500 steps of steepest descent and 9500 conjugated gradient minimizations), simulated annealing technique was used in order to explore the conformational space of the peptides with the following protocol. The system was heated from 300 K to 1000 K

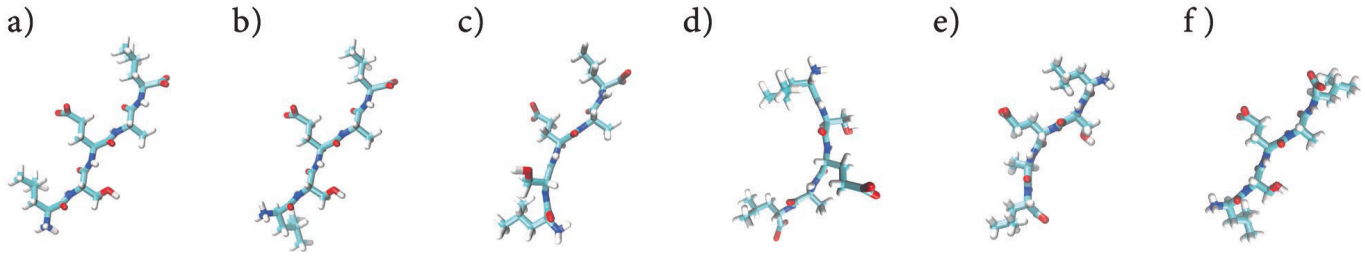

Figure 3: Structural representation of LSEAL in all 6 configurations. a) LSEAL in wild type configuration. b) lSEAL with Leu-1 in D-configuration. c) LsEAL with Ser-2 in D-configuration. d) LSeAL with Glu-3 in D-configuration. e) LSEaL with Ala-4 in D-configuration. f) LSEAl with Leu-5 in D-configuration.

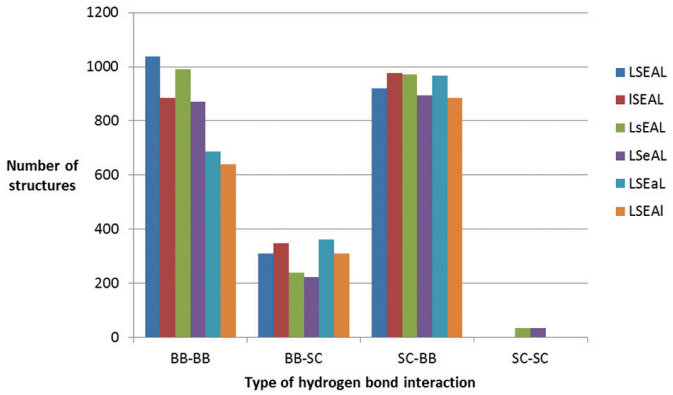

Figure 4: The number and types of hydrogen bonds observed within 3000 structures, for LSEAL wild-type peptide and its 5 configuration epimers. Interactions between atoms of the peptide backbone, as well as interactions between side chain atoms and atoms in the backbone make up the majority of the interactions found. Interactions between two atoms both found in the side chains are very rare.

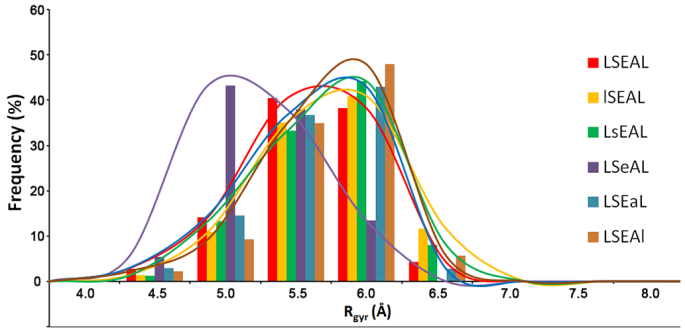

Figure 5: Radii of gyration (in Å) of wild-type LSEAL pentapeptide and its 5 epimers. The LSeAL epimer, with Glu-3 epimerized to the D-configuration, possesses the smallest average radius of gyration at 5.1 Å. The wild-type, in comparison, has a larger average radius at 5.4 Å and thus is a less compact molecule.

over 1000 femtoseconds (fs), and equilibrated at this temperature for an additional 4000 fs. The peptides were then cooled in a series of increments: first from 1000 K to 500 K over 1000 fs, then from 500 K to 200 K over 2000 fs, and finally from 200 K to 50 K over 7000 fs. This series of temperature increases and decreases was repeated a total of 3000 times, each yielding a unique peptide conformation. All 3000 conformations were then minimized using a combination of the steepest descent (ran for 500 steps) and conjugated gradient techniques (ran for 9500 steps).

The structural analysis of the conformations was performed with the *Ptraj* module of AmberTools12 software. The H-bonds (defined by a non-covalent bond length of <3.5 Å and donor-hydrogen-acceptor bond angle of >100°), secondary structure elements (via DSSP algorithm), and radius of gyration (R_{gyr}) were also calculated with the same software [17].

It should be noted here that statistical analysis was not preformed on the numerical results obtained as the present study is theoretical, and conducted entirely *in silica*.

Results and Discussion (Molecular Mechanics/ Molecular Dynamics)

1. Hydrogen Bonds

Within the six configurations of LSEAL (Figure 3), there are 24 possible pairings of donor and acceptor atoms involved in hydrogen bonds. The atoms involved in hydrogen bonding can be categorized into 2 major categories, atoms within the backbone chain (BB) of the pentapeptide and those within the side chains (SC) of the pentapeptide. The interactions involving two atoms both of which can be found within the carbon backbone (BB-BB) make up 50% of the possible pairings of atoms. In contrast, only one possible interaction involving two atoms in the side chains (SC-SC) is found across all epimers, between the serine residue at position 2 (Ser-2) and the glutamic acid residue at position 3 (Glu-3). The Glu-3 side chain involved in SC-SC interaction contains both carbonyl and hydroxyl groups while the Ser-2 only contains a hydroxyl group, thus Ser-2 acts as hydrogen donor and Glu-3 is the acceptor. One interesting finding is that the number of hydrogen bonds between Ser-2 and Glu-3 increases when either residue is epimerized to D-configuration (Figure 4). This is because epimerization of either Ser-2 or Glu-3 will result in a peptide with Ser-2 and Glu-3 side chains in closer proximity, leading to the increased possibility of hydrogen bond interaction between the only polar residues of the pentapeptide. However, the occurrence of SC-SC interaction is still quite rare, since at most only 1.13% of all 3000 structures tested exhibited this hydrogen binding pattern. This demonstrates the tendency of glutamic acid's large polar side chain to projects out of the peptide away from opposite of the other side chains in attempt to decrease hindrance and increase stability.

Glu-3(BB) appears to be mostly involved as an electron acceptor in the wild-type structure while Ser-2(SC) appears to be the most effective electron acceptor in other epimeric configurations. Leu-5(BB) and Glu-3(BB) show the highest tendency to act as hydrogen donors. This result may be contrary to expectation in that the central position of Glu-3(SC) and its functional groups would allow for higher probability of hydrogen bond formations within the side chain itself. The tendency of Glu-3 to project out of the folded structure, results in decreased hydrogen bonding interactions of the residue.

Out of 24 different hydrogen-bond pairings, 50% of the possible pair combinations are present in all six configurations (Figure 3). These common hydrogen bonds are therefore assumed to make no significant contribution to the changes in structural motifs and peptide folding in the event of epimerization. Moreover, the other half of hydrogen bonding combinations is likely to be crucial in determining the various secondary structures. This can be observed most visibly in the LSeAL epimer (lowercase notation represents an epimerized residue. In this case, Glu-3 is in D-configuration), which appears to have the most diverse combinations of atoms involved in hydrogen bonds, and was also observed to possess the largest variety of secondary structures.

2. Radius of Gyration

The radius of gyration (R_{gyr}), the root mean square distance of an object's extremities from its center of gravity or an arbitrary axis, is a measure of the compactness of an object. The R_{gyr} distribution, or the percent frequencies of average size, of the 3000 molecules in different conformations of each of the six configurations are depicted in Figure 5. Most of the wild-type LSEAL structures have an approximate R_{gyr} of 5.5 Å, with a smaller portion having R_{gyr} of ~6.0 Å. Relative to the wild-type, the peak of the LSeAL configuration was

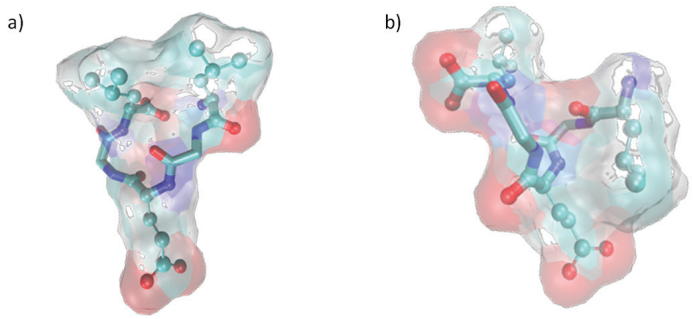

Figure 6: A molecular structure comparison between LSeAL and LSEAL pentapeptides. a) LSeAL pentapeptide has Glu-3 in D-configuration, and is more compact with an average R_{gyr} of 5.1 Å. The side chain of Glu-3 can be seen projecting away from the other amino residues, in order to decrease steric hindrance and stabilize the peptide structure. b) LSEAL pentapeptide in its wild-type configuration is less vigorously folded in comparison with an average R_{gyr} of 5.4 Å. The side chain of Glu-3 is oriented closer to the other residues.

found to be much lower, at ~5.0 Å, whereas all other configurations were shown to have relatively larger radii, predominantly ~6.0 Å. The differences in R_{gyr} between configurations could be explained by the functional groups of residues exerting attractive or repulsive forces onto each other, affecting the conformations of neighboring residues and the stability of the overall structure.

The LSeAL epimer (Figure 6a) was visually inspected to crosscheck the R_{gyr} results and was found to be more compact than the wild-type (Figure 6b). Both figures show the intramolecular interactions between Leu-1, Glu-3, and Leu-5. The chirality change of Glu-3 is shown to increase the number of secondary structure elements in the LSeAL configuration due to the large glutamic acid residue facing an opposite direction, eliminating the barrier between Leu-1 and Leu-5. Comparing this to the LSEAL configuration, the same interaction is not present due to the positioning of Glu-3 between Leu-1 and Leu-5. Glu-3 is observed to interact with both terminal Leu residues, diminishing the intramolecular interactions between the two. From these observations, we suggest that the central position of the Glu-3 allows it to have a relatively greater

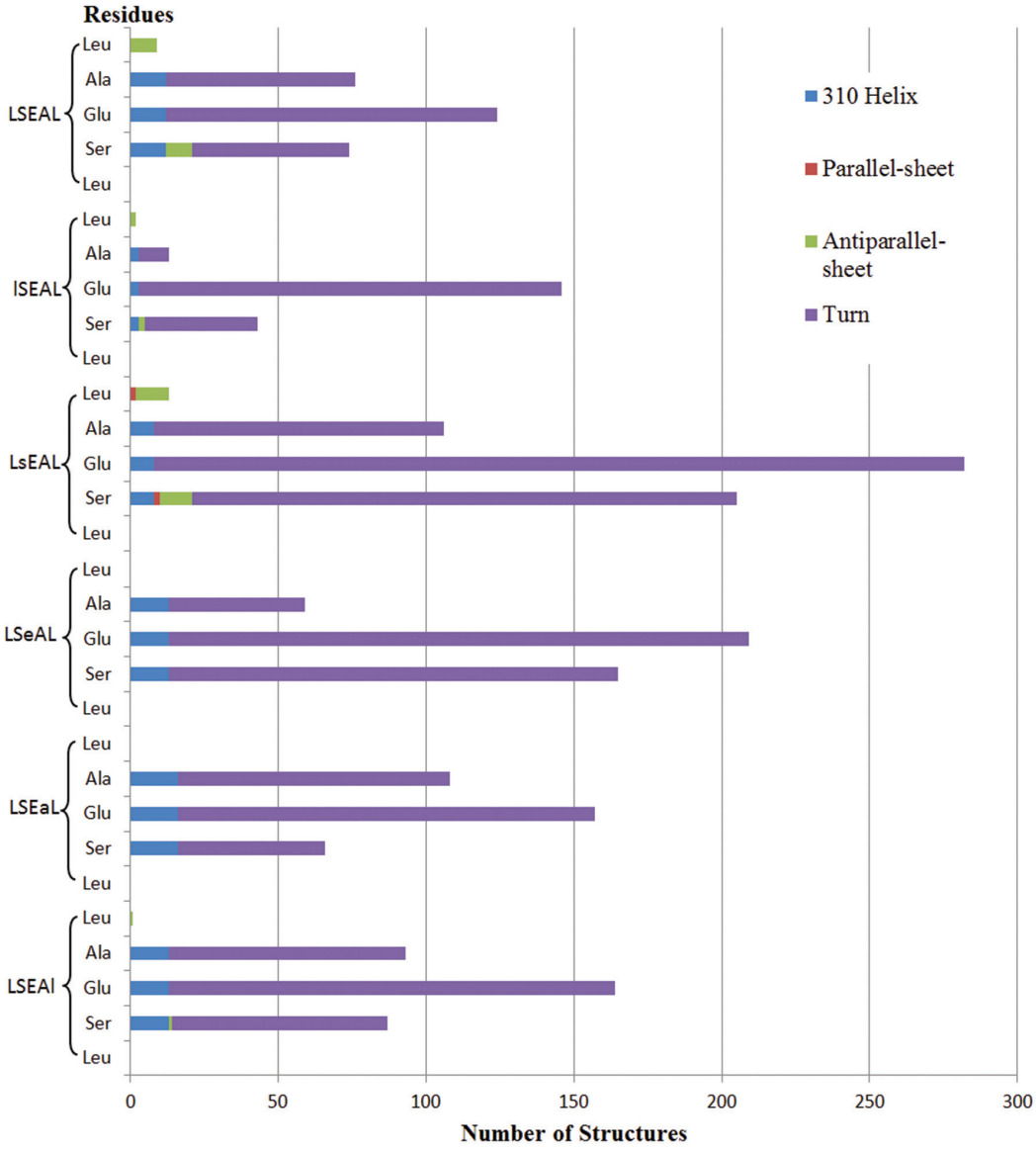

Figure 7: The number of each type of secondary structures, within the 3000 structures sampled for each of the six LSEAL pentapeptides in different configurations. The most prominent secondary structure present in all configurations is the turn structure, while only a few 3_{10} helix structures are observed.

potential to affect the overall structure of the peptide if epimerized. This effect on structure is consequently extended to the function of the substrate. Therefore, oxidization of this residue results in the most potential damage to the regular function of this substrate.

3. Secondary Structure

A total of four types of secondary structure elements were observed from the six epimers of the LSEAL pentapeptide (Figure 7).These four types are 3_{10} helices (a type of helix more elongated than an alpha-helix, in which every turn is completed by four amino residues), parallel and anti-parallel beta-sheets, and turns. Out of the secondary structures observed, the turn structures and the helices were the most abundant – found within every configuration. Turns were found to have the highest frequency of 15-20% in all epimers (Figure 7), but it is possible to interpret them as associates of the 3_{10} helices. This is because turns are defined as the close proximity of two residues that are three or four amino acids apart on the peptide chain, and the residues involved in turns are the same residues taking part in the formation of the helix. Each turn can be seen as the horizontal superimposition of the fourth residue onto the first in the 3_{10} helix and completing an entire 360° turn with every 3 residues (Figure 8). Therefore, it can also be said that the peptide occurs as an extended helix 15-20% of the time. On the other hand, approximately 80% of the residue inspected did not exhibit characteristics of any secondary structures, suggesting that most of the epimerized structures present in a biological setting can potentially alter their dihedral angles spontaneously.

It is also hypothesized from the results that the anti-parallel motif occurs in most of the epimers, as a result of the peptide strand completing a turn and commencing to run in the opposite direction. In contrast, the parallel structure requires an extra turn allowing the strand to continue in its original direction, forming a scheme (such as that in LSeAL) similar to a parallel beta-strand.

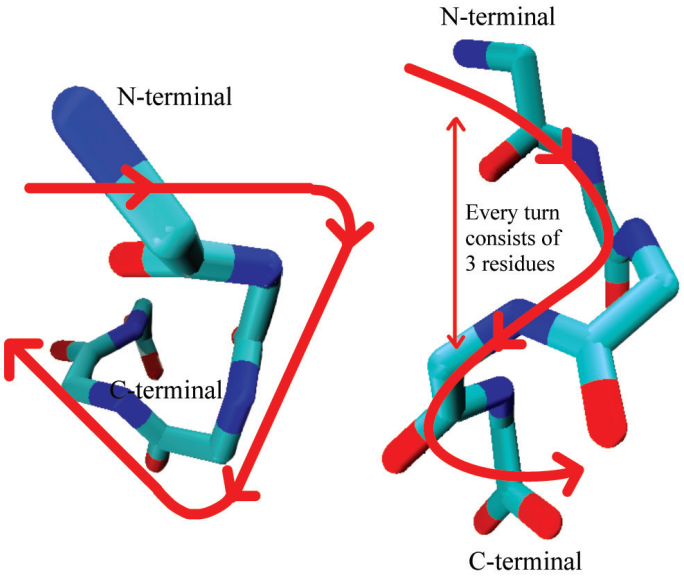

Figure 8: The 3_{10} helix structure of the wild-type LSEAL (top view, side view) is easily visible upon inspection of its molecular structure. The middle three residues (residues Ser-2, Glu-3, and Ala-4) complete a full 360° cycle. It can be observed from the side view that residues Ser-2 and Leu-5 overlap vertically, and this proximity is detected as a turn structural element in the calculations.

LSeAL was found to be the only epimer possessing parallel-sheet elements within its structure, which could be related to the many types of possible hydrogen bonding combinations within the epimer. Due to the restricted length of the pentapeptide, the occurrence of a parallel motif in addition to the anti-parallel requires a more twisted and compact structure, leading to the belief that the LSeAL configuration is more compressed and has a shorter molecular radius. However, since the number of anti-parallel structures observed in Figure 7 is very small proportional to the 3000 structures examined, more calculations need to be performed before further conclusion could be drawn.

Another phenomenon, shown in Figure 7, is the lack of structural involvement of the Leu-1 and Leu-5, which can be explained by the short peptide length and their terminal positions. The second, third and fourth residues are attached to amino acids on either sides, allowing an easy derivation of their secondary structures. In contrast, the first and fifth amino acids have only one neighboring residue that aids in the identification of their secondary structures, which is insufficient for the detection of possible secondary structures.

Although the results for parallel and anti-parallel sheets were deemed insignificant for conclusions, they still may be utilized to assess important structural modification for some of the epimers. For example, the LSeAL epimer is shown to contain all four structural motifs, from which it can be inferred that the epimers contains a large number of hydrogen bonds which may fold and compress the epimer structure, potentially disturbing peptide function. Overall, the LSEAL peptide generally assumes a 3_{10} extended helical structure in vacuum. This finding corresponds well with Deshmukh's research [7], which demonstrated that the twisted form of the pentapeptide is found to have higher concentration in the cytoplasm.

Conclusion

Oxidative stress in the body, caused by accumulation of ROS as aging occurs, may contribute to the disruption of normal cellular functions. This paper has explored the general effects of epimerization of individual residues on the overall LSEAL pentapeptide structure. A radical-induced epimerization yielding a D-configured amino acid can lead to changes in the pentapeptide conformation, affecting its affinity to calpain's binding site. Most noticeably, in LSeAL configuration, the epimerization of Glu-3 allowed for more potential interactions between the residues, resulting in the most drastic change in radius of gyration. Since the binding of LSEAL to calpain is largely dependent upon its structure and size, the small LSeAL structure may have lower affinity because it may slide through the binding site [7]. In vitro experiments can be used to further explore the consequences of residue epimerization, as the authors recognize that results from theoretical simulations will differ slightly with those obtained in a lab setting due to extraneous interactions with solvent and other solutes innately present in biological systems.

It is hoped that the results from this study may serve as a basis for future research on the binding mechanisms of LSEAL with calpain, as well as the inability of deformed epimers to bind to calpain due to chirality reversal by oxygen radicals. Further research may be done on the thermodynamics of the epimerization of LSEAL in order to get an insight into the energetics of the molecule and confirm the

result of the paper. This is particularly heightened in importance considering the potential for LSEAL to be used a therapeutic tool as a calpain inhibitor. Since hyperactivation of calpain causes the breakdown of neural cytoskeleton, such an inhibitory a tool would revolutionize the treatment of neurodegenerative diseases including Alzheimer and Parkinson disease [3].

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JULS RESEARCH

In Vitro Combination Treatments involving Curcumin and Resveratrol Produce Significant Anti-Cancer Effects on MIA-PaCa-2 Pancreatic Cancer Cells

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Abstract

Pancreatic cancer is one of the deadliest types of cancers, with a median survival time of 6 months. Over the past decade, numerous dietary compounds have been shown to possess anti-cancer properties against pancreatic cancer cells, both in vitro and in clinical studies. However, there have been few studies that investigated the effectiveness of treatments involving combinations of dietary compounds on pancreatic cancer cells. Two of the most promising dietary compounds are curcumin and resveratrol, commonly found in turmeric and red grapes, respectively. This project assessed the effectiveness of combination treatments involving curcumin and resveratrol, both of which possess relatively unique mechanisms of action, on MIA PaCa-2 pancreatic cancer cells. First, a dose response was conducted by assessing the cell number and cell metabolic activity (via the Alamar Blue assay) 72 hours after treatment under different concentrations of the individual compounds. Next, a combination treatment involving selected concentrations around the ED50, the dose which produces an effect in 50% of the population, of each compound was conducted and the effectiveness was measured using the same metrics. Our results show that this combination treatment produced a synergistic effect in inhibiting cell proliferation and survival, and an additive effect in inhibiting cell metabolism. These effects are significantly more effective on this pancreatic cancer cell line than individual treatments alone. This study illustrates that combinations of dietary compounds that possess different mechanisms of action are a promising new avenue of cancer research. Further studies could lead to the development of new therapeutic approaches to treat pancreatic cancer, such as novel diet supplements.

Introduction:

Pancreatic cancer is a lethal and aggressive malignancy, with a very poor prognosis. With a mortality rate that is almost identical as the rate of diagnosed cases (in the United States, there were 33,730 new cases and 32,300 deaths in 2006), it is the fourth leading cause of cancer-related deaths in the United States [1]. The median survival time is 6 months and less than 5% of patients live more than 5 years after being diagnosed with pancreatic cancer [1]. The poor prognosis stems from a lack of early detection and poor responses to current treatments [2]. Gemcitabine, the current treatment approved by the U.S. Food and Drug Administration, improves median survival by only a month [3]. Therefore, to decrease mortality rates and extend the lives of those with pancreatic cancer, new and more effective treatments are needed.

Certain dietary compounds may offer the potential for better treatments of pancreatic cancer. Resveratrol and curcumin are among the two most promising candidates to date. Resveratrol, a constituent of grape skins, berries and red wine, has been shown to block sulfonylurea receptors (SUR), regulatory subunits of ATP-sensitive potassium channels, and induce apoptosis in a SUR subtype-specific manner [4]. In addition, resveratrol has also been shown to upregulate macrophage inhibitory cytokine-1, which inhibits growth of pancreatic cancer cells [5]. Resveratrol has also shown to be effective in inhibiting pancreatic cancer stem cells, which arguably may be the most important targets in treating cancer [6]. When combined with gemcitabine, resveratrol also has proven to be more effective in tumor suppression than gemcitabine alone in mice models of human pancreatic cancer [7].

Curcumin, the active compound in the common spice tumeric [Curcuma longa], has been shown to decrease pancreatic cancer cell growth by inhibiting nuclear factor-kB [8]. NF-kB, active in pancreatic cancer cells but not in non-tumorigenic pancreatic epithelial cells, has been linked with cell proliferation, metastasis, angiogenesis and the inhibition of apoptosis [8]. In addition, combined with gemcitabine, curcumin has been shown in mice models to be effective in reducing angiogenesis and decreasing tumor size compared to gemcitabine alone [8]. Moreover, curcumin has been shown to target tumorigenic pathways by irreversibly binding to CD13/aminopeptidase N (APN), inhibiting the signal transducer and activator of transcription (STAT) proteins, inhibiting the peroxisome proliferator-activated receptor-g (PPAR-g), and downregulating the expression of COX-2 protein] [9].

Unlike gemcitabine, which has shown to possess significant bone-marrow and pulmonary toxicity [10], dietary compounds are very safe for consumption in comparison. For example, clinical studies have shown that curcumin is safe even when consumed at 12g per day for 3 months [9]. As a result, effective dietary compounds may serve as superior alternatives to current pancreatic cancer treatments.

Although the two dietary compounds individually have shown promising results, there has been no study to date on the effects of combination treatments of resveratrol and curcumin used together on pancreatic cancer cells. Since the two dietary compounds both have anti-tumor properties with unique mechanisms of action, it is hypothesized that a combination treatment involving these compounds may yield an additive or synergetic effect. Thus, this study aims to see if combinatory treatments will have a greater effect on pancreatic cancer, specifically, the pancreatic cancer cell line MIA PaCa-2, than individually. If synergistic or additive effects are seen in vitro with combination treatments, these compounds and other similar phytochemicals could potentially offer novel and more effective treatments for pancreatic cancer patients to improve their prognoses.

Materials and Methods

Cell Culture

MIA-PACA2 cells were cultured using standard mammalian cell culture techniques. The cells were thawed from storage, cultured in T-25 flasks (Corning) using a medium consisting of 89% Dulbecco's Modified Eagle Medium [Sigma-Aldrich Canada), supplemented with 10% Fetal Bovine Serum(Sigma-Aldrich Canada] and 1% Penicillin and Streptomycin (Sigma-Aldrich Canada). The cells were maintained in a 37 °C degree incubator at 5% CO_2. The cells were passaged using Trypsin (Sigma-Aldrich Canada) twice per week.

Dose Response:

A dose response was conducted for curcumin and resveratrol to confirm the results from previous studies, to establish the concentrations required for the combination treatments and to establish baselines to compare with combination treatments. Three concentrations for each compound similar to those used in published studies were tested in the dose response. For curcumin, 5μM, 10 μM and 20 μM were tested. For resveratrol, 10 μM, 20 μM and 50 μM were tested. Stock solutions of curcumin and resveratrol were retrieved from and serially diluted in dimethyl sulfoxide [1:1000] to the required treatment concentrations. The treatment medium consisted of DMEM, 2% FBS and 1% Pen/Strep along with the treatment compounds. A treatment medium for the control and a solvent control were also made.

Two 24-well plates were labelled, one for the resveratrol and one for curcumin. In each 24-well plate, five columns of triplicate wells were labelled. The first column of triplicates was labelled as the control, the second column as the solvent control (DMSO was used as the solvent), the third column as the first treatment concentration (5μM for curcumin and 10μM for resveratrol), the fourth column as the second concentration (10μM for curcumin and 20μM for resveratrol) and the fifth column as the third concentration (20μM for curcumin and 50μM for resveratrol). See Figure 1 for a schematic of the set-up.

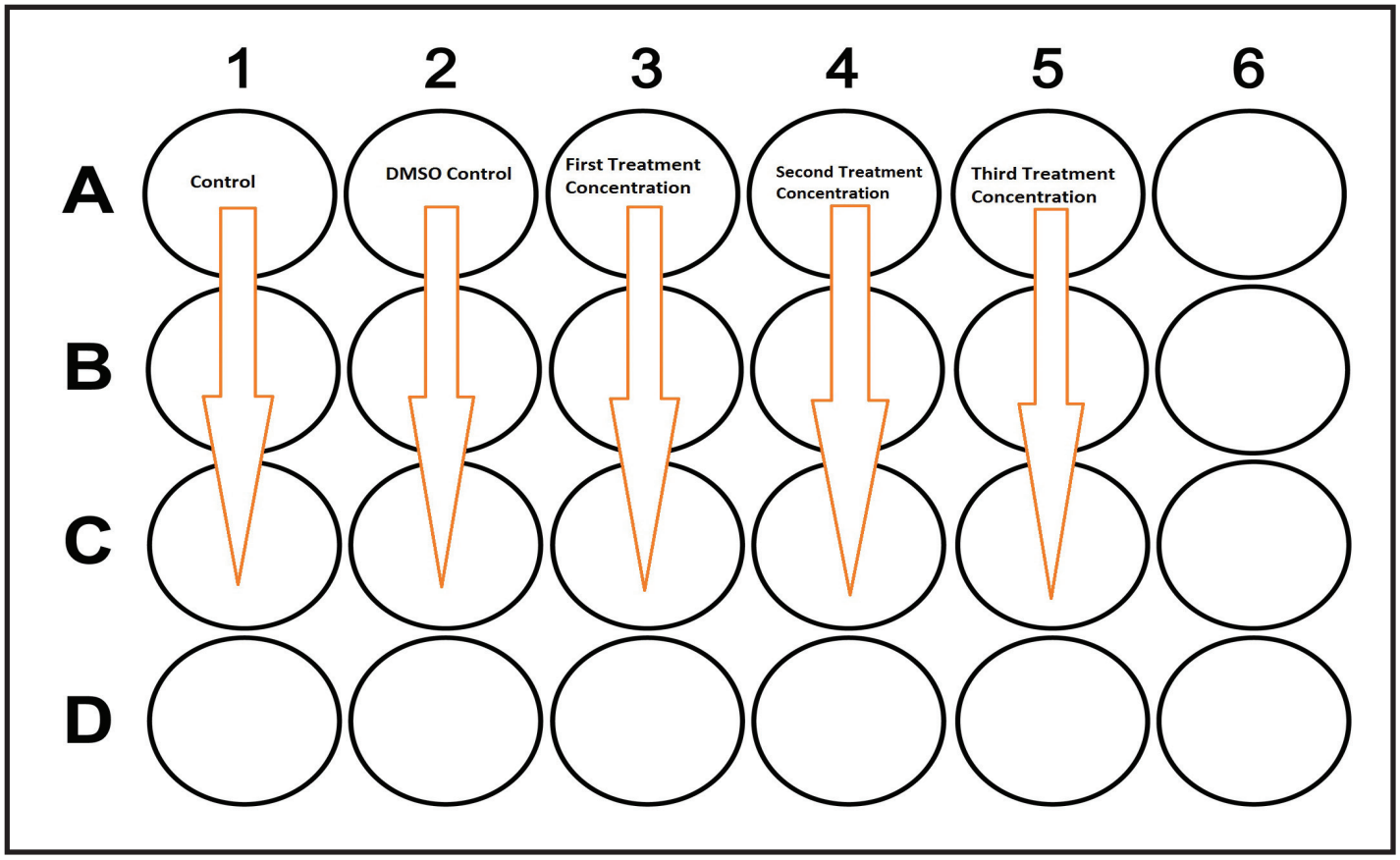

Figure 1: Schematic of Dosage response set-up

1mL of $2.5x10^4$ cells/mL MIA-PACA2 cells, suspended in the culture medium, were seeded in each of the labelled wells. After 24 hours to ensure cell attachment, the culture medium was aspirated and replaced with the treatment media. After 24 hours of treatment following media exchange, the treatment media were aspirated and replaced with fresh treatment medium. After 24 hours of additional treatment (48 hours treatment total), Alamar Blue assay and direct cell counting were performed to assess cell health and viability.

Alamar Blue Assay:

100uL of Alamar Blue reagent(Molecular Probes) was added to each well and incubated in darkness as Alamar Blue is photosensitive, and placed into the 37 C, 5% CO_2 incubator for 3 hours. After incubation, the well plates were put into a microplate reader (Thermo Scientific) and the amount of fluorescence emission was measured and recorded. The emission of the plate reader was set to 590 nm, the excitation was set to 550 nm, and the cutoff was 570 nm, as recommended in the Alamar Blue reagent guidelines.

Direct Cell Counting:

The cells were trypsinized, centrifuged and re-suspended in culture media. Small aliquots of re-suspended cells were placed into micro-centrifuge tubes and Trypan Blue (Gibco) was added in a 1:1 ratio to distinguish live and dead cells. The cells were counted using a Bright-Line hemacytometer (Sigma-Aldrich) under a phase-contrast microscope.

Combination Treatments:

Based on the dose responses, concentrations around the ED50 of each compound were selected for the combination treatment. The same procedures described in the dose response section were used to assess the effectiveness of a combination treatment involving 5μM of curcumin and 10um of resveratrol. Alamar Blue assay and Direct Cell counting were used as described above.

Statistical Analysis:

Statistical analysis, namely a t-test and calculating the standard errors, were performed on Microsoft Excel 2010. The graphs were also generated using Microsoft Excel.

Results

As expected, Alamar Blue results and direct cell counting results show that combination treatments involving curcumin and resveratrol are significantly more effective on MIA PaCa-2 cells than individual treatments at the same concentrations (Figures 2 and 3). Using the Alamar Blue assay, the combination treatments involving curcumin and resveratrol produced an efficacy (as measured from fluorescence intensity) somewhere between the results for doubled doses of the respective compounds (as determined by the dose response), indicating an additive effect on metabolic activity (Figure 2). For the direct cell counting results, the combination treatments suggest synergistic effects since the numbers were significantly reduced (Figure 3). All results had a p value of less than 0.1. However, the authors predict that if the sample size was bigger, the p values would have been even smaller than those seen.

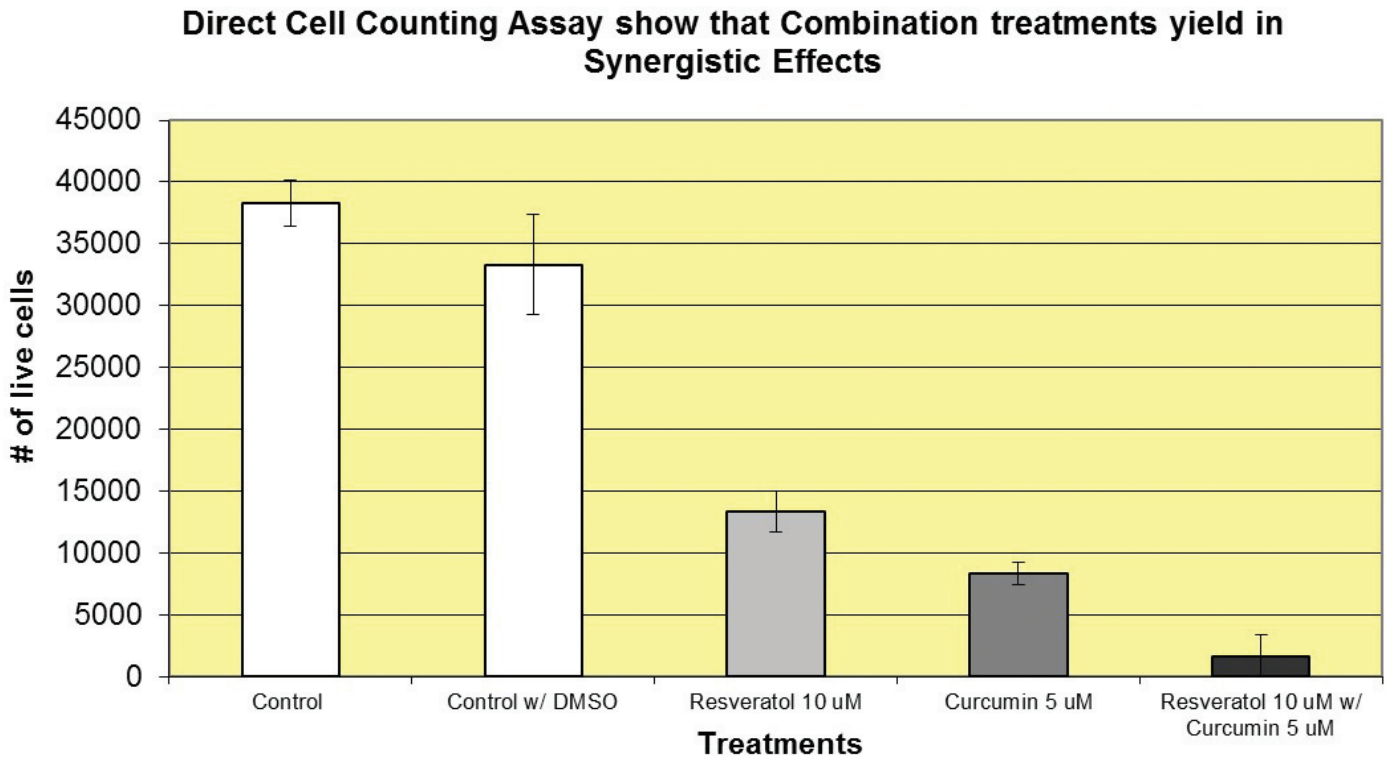

Figure 2: Direct Cell Counting Results show combination treatments yield in synergistic effects in cell proliferation and survival. T-test: Control+DMSO vs Res 10 p=0.09965, Control+ DMSO vs. Cur 5 p=0.03521, Control+DMSO vs. Cur5+Res10= 0.0071, Res 10 vs. Cur5+Res10 p=0.0728, Cur 5 vs. Cur5+Res10 p=0.0814

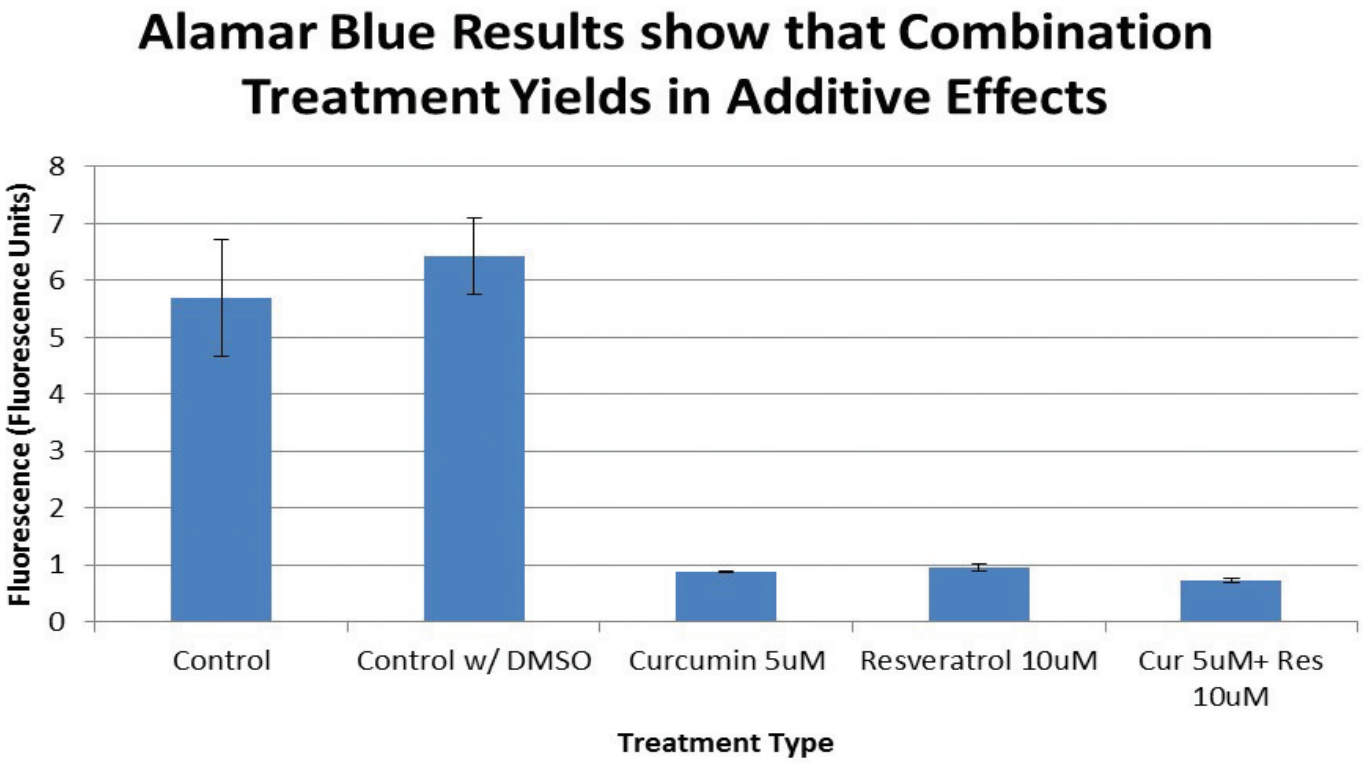

Figure 3: Alamar Blue Results show that combination treatment yield in additive effect in cell metabolism. One-Way ANOVA: Control+DMSO vs Res 10 p=0.0144, Control+ DMSO vs. Cur 5 p=0.0139, Control+DMSO vs. Cur5+Res10= 0.00137, Res 10 vs. Cur5+Res10 p=0.0773, Cur 5 vs. Cur5+Res10 p=0.0184

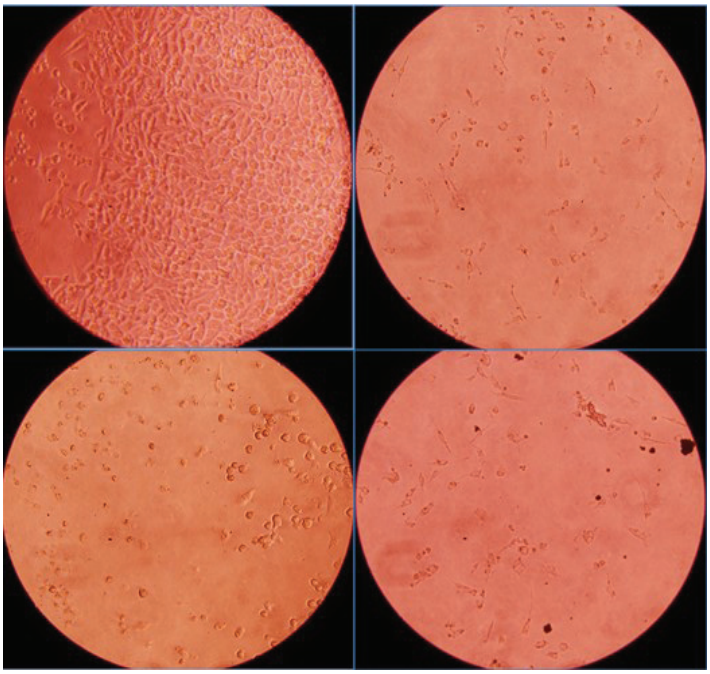

Figure 4: Images 72 hours after treatment. Top left is Control, top right is Res 10, Bottom left is Cur 5, Bottom right is Cur5+Res10

Discussion

This study shows that the combination of resveratrol and curcumin yielded promising results in inhibiting pancreatic cancer cell growth and proliferation. However, since MIA PaCa-2 may not be completely representative of pancreatic cancer, it is necessary to conduct similar studies on a panel of pancreatic cancer cell lines, such as Bx-PC 3, Panc-1 ASPC-1 and Hs-766t, which have been previously studied [11]. Another logical next step would be to conduct combination studies involving resveratrol and curcumin on pancreatic cancer stem cells, which may be vital to treating tumors. After such in vitro studies have been conducted, similar combination treatment studies should be tested on a mouse xenograft model of human pancreatic cancer via oral administration, as in vivo models are more far representative of actual disease.

In addition, population studies have shown that resveratrol and curcumin may be individually effective in preventing pancreatic cancer [12,13]. However, no study has assessed the effectiveness of the combination of the two on preventing pancreatic cancer. Thus, long-term population studies should be done to determine if the combination of resveratrol and curcumin has any preventative value, by assessing if people who have diets containing significant amounts of both compounds have lower incidences of pancreatic cancer. Similar combination population studies should also be conducted using other combinations of dietary compounds.

The results from our study further add to the results from a growing body of studies that show the promise of combination treatments involving dietary compounds (ex. catechin and IP6, EGCG and zVad curcumin and isoflavone, DHA and curcumin) [14,15,16]. Further research on combination treatments involving dietary compounds could lead to the development of treatments that are safer and more effective than gemcitabine, the current standard for treating pancreatic cancer. In addition to treatment, such therapeutic approaches, which may include simple dietary changes, may prove to be effective in the prevention of pancreatic cancer.

Conclusion

This study investigated the effectiveness of combination treatments involving resveratrol and curcumin, two dietary compounds that have individually shown to be effective against pancreatic cancer. The two compounds were selected as they possess unique mechanisms of action. The results shown from the Alamar Blue assay and direct cell counting show that combination treatments involving curcumin and resveratrol are significantly more effective on MIA PaCa-2 cells than individual treatments at the same concentrations. Specifically, the results show that this combination treatment produced a synergistic effect in inhibiting cell proliferation and survival, and an additive effect in inhibiting cell metabolism. Further studies involving combination treatments of dietary compounds could lead to safer and more effective treatments against pancreatic cancer.

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JULS REVIEWS

Influence of Oxytocin on Prostate Cell Proliferation and its Potential Role in Cancer Treatment and Prevention

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Abstract

Recent research has shown that oxytocin may be a treatment for prostate cancer. Oxytocin regulates the activity of 5-α-reductase isoenzymes, which reduces dihydrotestosterone to testosterone in the prostate. By using oxytocin to control this enzymatic activity, inhibition of prostate cell proliferation can be achieved to suppress cancerous growth. However, other studies report that oxytocin levels in benign prostatic hyperplasia (BPH) patients are much higher than normal. This suggests that oxytocin could also promote cell growth and cancer development as well. Experiments *in vitro* suggest that more gene expression of oxytocin occurs in prostate cancer cells than in the normal prostate cells, and oxytocin may induce cell migration in prostate cancer cells. Moreover, other studies report that oxytocin's influence on cellular proliferation is dependent on the location of the oxytocin receptor. Co-localization of the receptor with caveolar protein hcav-1 is believed to cause a change in secondary messengers of G-protein signaling. A change in the secondary messenger results in suppression of pERK downstream, a protein that can regulate mitotic division. Without the control provided by pERK, hcav-1's overall effect leads to increased prostate cell proliferation and cell growth. Further clarification is needed on the involvement of oxytocin in prostate cancer. The following review is focused on the influence of oxytocin on prostate cancer cells and the investigation of various potential treatments.

Introduction

Oxytocin is a nonapeptide neurohypophyseal hormone responsible for many biological processes in the human body including sexual reproduction, breastfeeding, social cognition, and emotional functions [1, 2]. Oxytocin receptors are present broadly within the central and peripheral nervous system, which reflects its various endocrine and paracrine activities [3]. Given the recent discovery of its involvement in type-dependent proliferation and anti-proliferation of cancer cells [4-7], oxytocin has been broadly studied as a treatment for cancer. However, these studies on oxytocin and its effect on prostate cancer have revealed contradictory results [8-10]. Oxytocin may be a potential treatment for prostate cancer, due to its ability to inhibit cancer cell proliferation by regulating the expression or activity of 5-α-reductase isoenzymes [11]. Conversely, oxytocin also appears to induce prostate cancer cell migration and proliferation through oxytocin receptor co-localization with caveolin proteins [12, 13]. The implications of oxytocin on the proliferation and anti-proliferation of prostate cancer cells will be discussed in detail.

Oxytocin's role in inducing 5-α-reductase activity

Normal growth and functions of the prostate require the presence of androgenic hormones and dihydrotestosterone [14]. Dihydrotestosterone is produced by reducing testosterone, and this process is catalyzed by 5-α-reductase isoenzymes type I and type II [15]. Type I is mostly found in fetal scalp and tissues of prostate and liver [16], whereas type II is mainly found in the internal genital tissues, including the prostate [17]. Both types are found in the stromal and epithelial cells of hyperplastic human prostate [18]. These two types are located on different chromosomes and they vary in kinetic properties [19]. In normal prostate tissue, type II has a higher affinity for testosterone than type I, and it is also expressed in greater level [20]. In 2008, a study done by Uemura *et al.* confirmed the existence of type III 5-α-reductase isoenzyme in prostate tissue at the mRNA level [21]. However the levels of expression and exact functions have yet to be discovered. The human benign hyperplastic and normal prostate express both oxytocin and oxytocin receptor in the epithelial and stromal cells [22, 9]. Data obtained from rat prostate has shown that oxytocin is able to increase the expression of 5-α-reductase type II and decrease the expression of type I in prostate tissues [23].

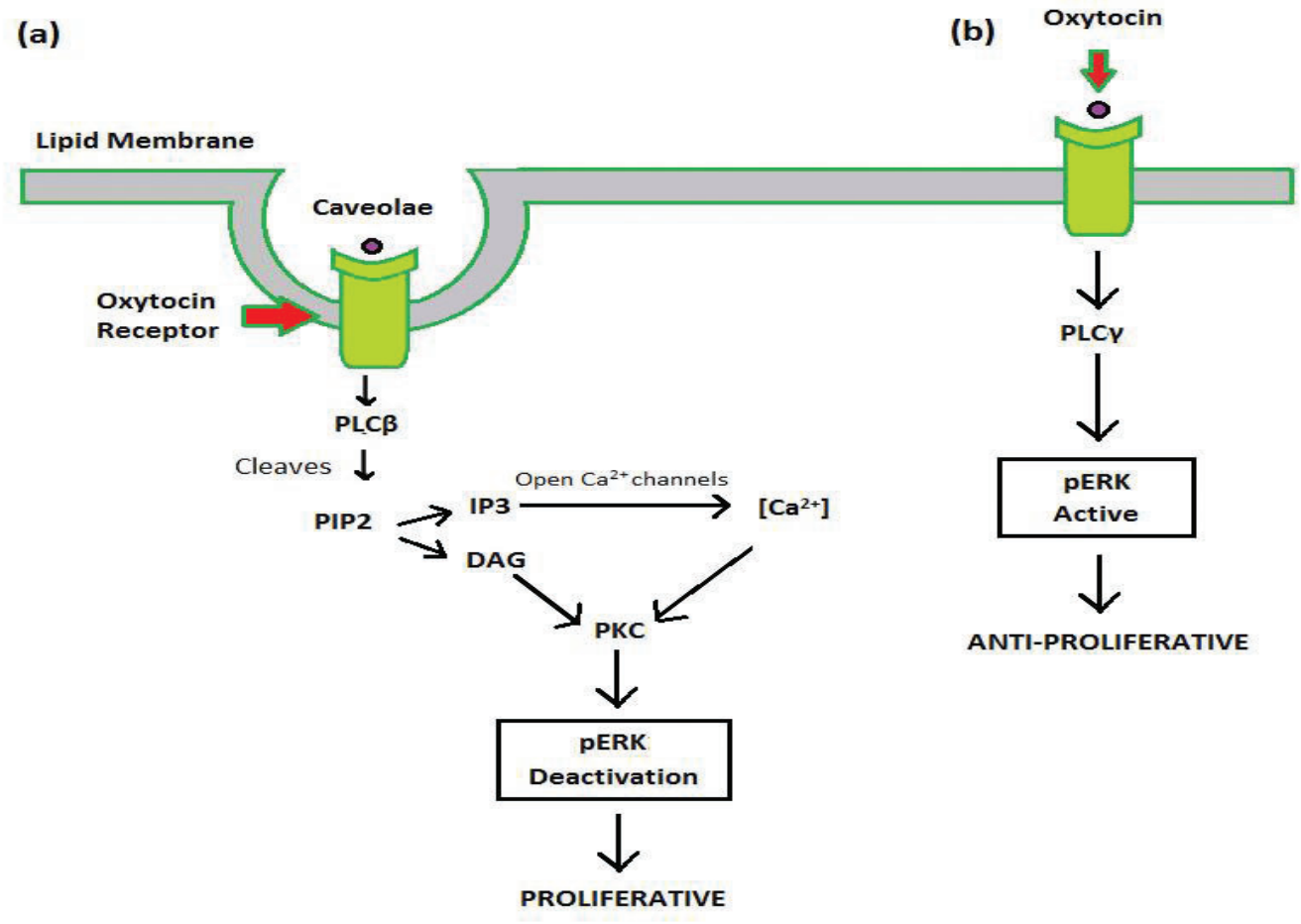

Figure 1: Downstream effects of oxytocin receptor are determined by receptor localization in and out of caveolar invaginations. Oxytocin regulatory effects on cell growth are dependent on the G-protein signalling pathways. (a) Oxytocin binding within caveolae leads to production of secondary messenger phospholipase C-β (PLCβ). PLCβ cleaves phosphatidylinositol 4, 5-bisphosphate (PIP2) into inositol 1, 4, 5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 binds to IP3 receptors, opening calcium ion channels. The calcium ions in the cytosol work with DAG to activate protein kinase C (PKC). PKC deactivated pERK, a suppressor of cell proliferation. (b) When oxytocin binding occurs outside the caveolae, phospholipase C-γ (PLCγ) is produced instead. PLCγ activates pERK, leading to transcriptional repression of proteins and reduced cell growth.

In a study conducted by Assinder and collaborators, a prostate cell culture with oxytocin plus oxytocin antagonist was incubated, while another prostate cell culture was incubated with only oxytocin. The efficiency of the conversion of testosterone to dihydrotestosterone was measured in the cell culture. Cells treated with only oxytocin had a significantly greater activity of 5-α-reductase than the cell incubated with oxytocin plus oxytocin antagonist [11]. Taken together, these results provide evidence for the modulation of the expression or activity of 5-α-reductase isoenzymes in the prostate by oxytocin.

Other studies suggest that oxytocin can be used for the treatment of prostate cancer by inhibiting the proliferation of prostate cancer cells through regulating the expression or activity of 5-α-reductase isoenzymes [24]. However, Assinder also showed in this study that if the concentration of oxytocin used was equal to or exceed 50nM, the difference in 5-α-reductase activity was not significant in comparison to the control samples. As a consequence, the regulation of 5-α-reductase isoenzymes by oxytocin is dose dependent. Overall both types of 5-α-reductase isoenzymes are expressed in human prostate epithelial and stromal cells, while oxytocin only regulates 5-α-reductase type I and II found in epithelial cells, not in stromal cells [11]. Consequently, oxytocin may not have a significant effect on the expression of 5-α-reductase isoenzymes type I and type II in prostate tissues.

Alternately, Nicholson has shown that oxytocin concentration in male prostatic tissues with benign prostatic hyperplasia (BPH) is much higher than in healthy men [25]. This indicates that a high concentration level of oxytocin may potentially lead to cancer. *In vitro* studies have shown that the secretion of oxytocin is regulated by estrogen and androgens. If the ratio of estrogen: androgen elevates, then the secretion of oxytocin increases as well [26]. However, increasing the amount of estrogen leads to the promotion of stromal cell proliferation, which is an initiator of the benign prostatic hyperplasia formation [27]. According to these results, oxytocin may also play a role in the aetiology of prostate cancer. Nonetheless, the role of oxytocin in prostate cancer is still unclear.

Oxytocin receptor's relationship to caveolin-1 protein and the caveolae folds

The prostate depends on testosterone and androgen binding proteins (ABP) for contraction, secretion, and cell growth. While past studies have shown that oxytocin has inhibitory effects on prostatic cell growth by regulating androgen concentration, recent observations show that the exact effects of oxytocin on prostate cell proliferation depend on its location within the lipid membrane [28]. Experiments have shown that oxytocin

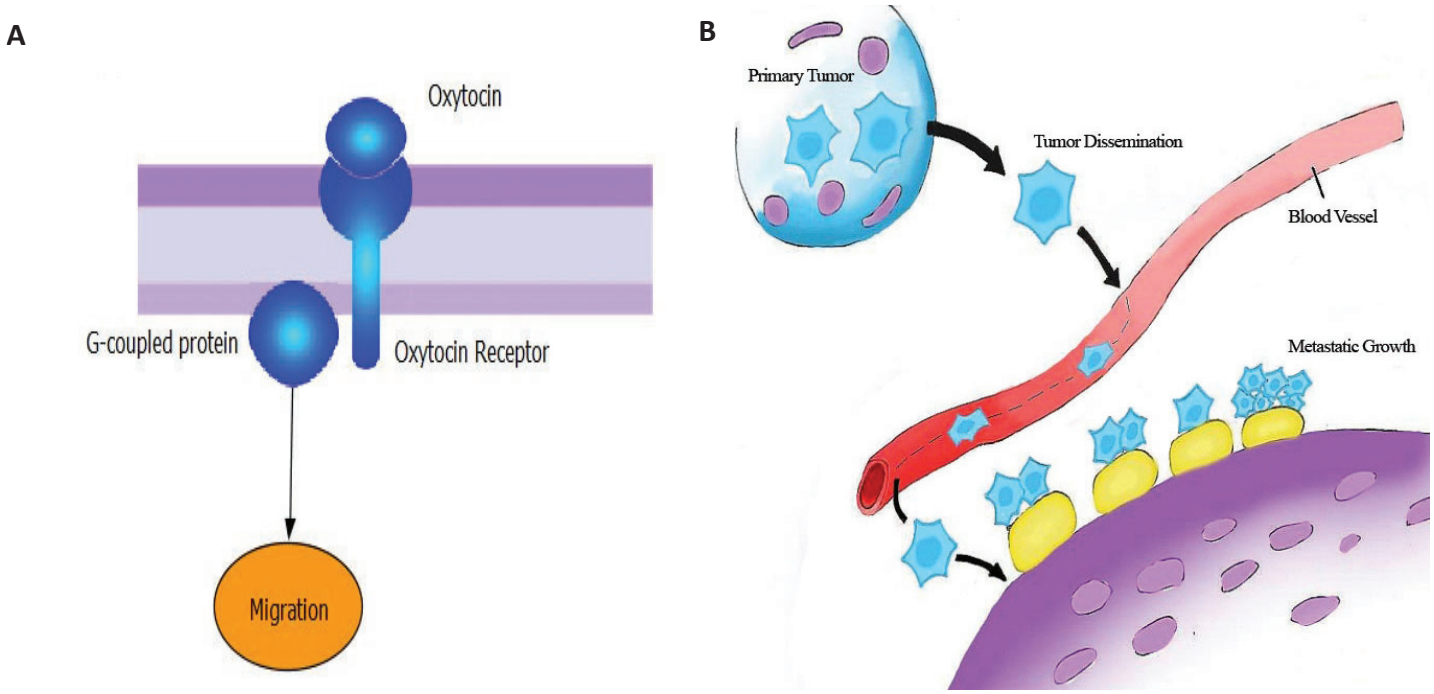

Figure 2: A) The binding of oxytocin to oxytocin receptor which activates the G-coupled protein, and thus induces cell migration [12]. B) Showing of tumor cell migration and metastatic process. The invasive cancer cells migrate from the primary tumor and penetrate the neighbouring blood vessel. The cancer cells are transported by normal blood flow to a distant organ, and the metastatic growth within the distant organ results in the formation of a secondary tumor site. Further spread of cancer cells can trigger death in cancer patients.

receptors can both stimulate or repress prostate cell growth depending on its location inside or outside regions of membrane invaginations, called caveolae [29].

Within the caveolae, the cell membrane is enriched with sphingolipids, cholesterol, and various proteins. The protein caveolin-1 (hcav-1) in particular is the most abundant in the caveolae [30]. Through the use of immunoelectron microscopy, past observations have shown that expression of the protein hcav-1 strongly correlated to the number of caveolae found on the lipid membrane [31]. In contrast, gain and loss of other caveolae proteins, hcav-2 and hcav-3, did not change the number of caveolae observed [32].

Though the exact process is still unclear, the interactions between oxytocin receptors and the caveolae environment is believed to alter the oxytocin receptor signalling pathway, activating downstream effector phospholipase C-β instead of phospholipase C-γ (Figure 1) [33]. This change leads to deactivation of pERK, a transcriptional regulator, downstream by protein kinase C (PKC). By phosphorylating eukaryotic translation-initiation factor 2, pERK inhibits expression of proteins vital for mitotic division. With pERK deactivated by PKC, the regulatory mechanism is shut down and cells can proliferate uncontrollably. Thus, overabundance of caveolae resulting from increased hcav-1 expression may lead to irregular prostate cell proliferation, resulting in prostatic hyperplasia and potentially prostate cancer [13, 34].

Although hcav-1 is the major protein in caveolae and has a role in caveolae formation, a study led by Libin *et al.* suggests that another caveolar protein called Transcript Release Factor (PTRF) is required for caveolae to form [34]. A large number of hcav-1 proteins were found within PC3 cancer cell lines, and yet very few caveolae are present. Proteomic screens of mRNA in PC3 revealed that PTRF is not expressed. Separate experiments conducted by Hill *et al.* used mice models to illustrate that injection of PTRF into PTRF-deficient mice formed caveolae in the membrane [35]. Though these studies have suggested that lipid rafts have trouble forming caveolae without PTRF, it is still not clear whether the absence of PTRF occurs exclusively in cancer cells or if PTRF expression directly correlates with the progression of prostate cancer [36].

The results of Libin's studies with PC3 are contradictory with respect to pathways involved in oxytocin receptor localization. PC3 cell lines are cancerous, and yet they have fewer caveolae than normal prostate cells. As a consequence of PC3 cultures having fewer caveolae, OT receptors within the caveolae would either be less occurring, or be condensed into the few caveolae regions that exist. The latter can be ruled out if immunoprecipitation studies can show that the OT receptor does not have an affinity with caveolar proteins, and that there is no tendency for the receptor to migrate into lipid membrane invaginations.

In an *in vitro* experiment involving immunocytochemistry, the oxytocin receptor and hcav-1 proteins were located in human prostate samples. This is done by looking for specific immunoreactions inside the tissue when treated with hcav-1-binding and OTR-binding antibodies [37]. Surgical tissues were categorized into 3 groups based on the donors they were obtained from, consisting of young non-BPH donor tissue, old non-BPH donor tissue, and tissues from BHP patients of various ages. The results from the immunocytochemistry showed that 9 year olds had 18% occurrence of co-localization of hcav-1 and OTR, 28 year olds had 40% occurrence, and BPH patients had 62% occurrence [38]. Experimental data suggests that co-localization of hcav-1 with oxytocin receptors increased with age, and is strongly correlated to BPH. Although BPH does not lead to cancer, hyperplasia is a risk factor of prostate cancer. BPH and prostate cancer show many other similarities: they both have increased risks with age, and are clinically diagnosed using similar methods [39]. Though the exact relationship between the two is still unknown, both involve oxytocin receptor regulation.

Elevated level of oxytocin in prostate cancer cell migration

In various *in vitro* experiments, oxytocin acts as a growth inhibitor which inhibits the proliferation of various tumour cells including breasts, neural tissue, and bone cells [40-42]. Conversely, other studies have suggested that there is a correlation between the amount of oxytocin expression and the amount of small cell lung carcinoma [43], and it was also found to promote prostate cancer cell proliferation [44]. This section will focus on the evidence of oxytocin-induced prostate cancer cell migration.

Cell migration is an essential biological process for development and maintenance of multicellular organisms. However, the same processes used for cell migration during development and maintenance are also activated during metastasis to disseminate cancer cells (Figure 2B) [45].

Early prostate cancer cells require androgen to survive and proliferate by the activation of androgen receptor signalling [46]. Similarly, an increasing number of findings suggest the correlation between the presence of oxytocin/ oxytocin receptors found in various tumour cells and their growth [47]. In one study led by Zhong *et al.* on the relationship between oxytocin/ oxytocin receptor, and prostate cancer cell lines *in vitro*, eight prostate cell lines were examined, including PrEC normal prostate basal epithelial cells, RWPE1 immortalized prostate luminal epithelial cell lines, k-ras-transformed RWPE1, PrSC normal prostate stromal cells, and LNCaP, DU145, PC3, and PC3M prostate cancer cell lines [12]. The results showed that oxytocin and oxytocin receptor mRNA were present in all eight prostate cell lines, however, a relatively higher level of oxytocin receptor mRNA was found in prostate cancer cell lines, especially in PC3, and the highest amount of oxytocin peptide also occurred in the prostate cancer cell line lysates.

This data agrees with results from previous experiments on expression of oxytocin and its receptor in prostatic malignant tissues [47], but conflicts with the data provided by Whittington et al. and Farina-Lipari et al. in vitro which showed a loss of oxytocin expression with the progression of prostate cancer [10, 48].

Oxytocin receptor is a G-protein-coupled receptor which couples to multiple proteins including G_i and G_q heterotrimeric complexes and synergistically conveys oxytocin signalling to different intracellular pathways that lead to distinct cellular functions (Figure 2A) [49, 12]. The oxytocin-induced prostate cancer cell migration was completely blocked by treating the cell cultures with an G_i protein inhibitor, which suggests a role for the oxytocin receptor in prostate cancer metastasis, though the specific mechanism of the oxytocin-induced prostate cancer cell migration was not discussed in the study [12]. Likewise, in a different study by Reversi *et al.* on oxytocin, it suggests that oxytocin receptor- G_q signaling stimulates cell growth whereas the coupling of oxytocin receptor to G_i acts as an antagonist which inhibits cancer cell growth and migration promoted by oxytocin receptor-G_q signalling [49].

Prostate cancer cell migration was identified using cell migration assays, where oxytocin induced a dose-dependent increase in migration of PC3 and PC3M and induced a 2-fold increase in PC3M cells [12]. The stimulated prostatic cancer cell migration by oxytocin can play a potential role in dissemination and invading other distant organs, driving metastasis in vivo [45].

To summarize this section on the role of oxytocin in prostate cancer cell migration, the experimental data suggest that migration of prostate cancer cell lines PC3 and PC3M are stimulated by oxytocin, and more oxytocin peptides are found in prostate cancer cell lines, which might serve as a mechanism for prostate cancer cell progression [40, 50]. However, the explicit biological effect of the expression of oxytocin and its receptor mRNA found in prostate cancer cell lines has not been explained yet [12]. Therefore, the exact role of oxytocin in prostate cancer remains controversial due to the conflicting data in different experiments [10].

The future of research in oxytocin

Numerous experimental observations support that oxytocin receptor localization with hcav-1 may promote prostate cancer, but it still remains to be determined if the correlation between increased cancer risk and co-localization of the two have is a causal relationship. If the relationship exists, would other caveolae proteins such as hcav-2 and hcav-3 fulfill similar roles to hcav-1 in shifting the oxytocin receptor regulatory signalling pathways (Figure 1). Furthermore, the exact process that is inducing the co-localization in cancer cells still remains at large. It is possible that during cancer progression, the oxytocin receptor may develop an affinity for caveolae proteins, or have a structural tendency to be located in the caveolae folds.

Further experimental data on oxytocin influence on 5-α-reductase enzyme needs to be obtained, as the previous studies have shown contradicting results. Some suggest oxytocin can be used as a potential therapy for prostate cancer while others suggest that it may be the causing factor. The exact role of oxytocin on prostate cancer cells is yet unclear.

Continued research in the future is required to provide answers to all the questions raised in present studies and determine if oxytocin can lead to potential treatments for prostate cancer.

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JULS REVIEWS

The potential benefits of using RNA interference (RNAi) methods to lower viral replication and infectivity by targeting the *PB1* gene in influenza A

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Abstract

Every year, influenza continues to put a strain on global health by limiting hospital and financial resources and by reducing workplace productivity. Seasonal influenza places a major socio-economic burden on many countries. In the United States alone, billions of dollars are lost yearly to absenteeism from work, resulting in lowered productivity. Influenza A virus subtypes mutate to form different combinations of surface proteins hemagglutinin (HA) and neuraminidase (NA). As a result, different types of strains circulate throughout the population annually, weakening human immunity and increasing drug resistance. Human influenza viruses can re-assort, or mix genetic material, with avian viruses, forming new combinations of surface proteins. H5N1 avian influenza (AI) is known to cause serious infections and high mortality rates, estimated around 60% or higher. Since there is increasing concern over an imminent pandemic, it is necessary to adopt novel methods that lower virus replication and infectivity, which could be used further to create better antiviral therapies and vaccines. Recently, RNA interference (RNAi) methods have been used to inhibit the replication of the virus in the laboratory, using modified small interfering RNAs (siRNAs) targeting conserved regions of influenza genes through the natural RNAi gene-silencing pathway. Such RNAi methods could be used to specifically target the conserved PB1 subunit of the RNA polymerase in influenza A to lower viral replication, since PB1 is responsible for RNA polymerase's catalytic activity and viral replication. However, controversies remain surrounding the import, function, and structure of PB1. Having a more precise understanding of PB1 will help connect viral replication processes to the host immune response. Previous studies have demonstrated that viral replication can be reduced by RNAi-mediated knock-down of *PB1*. This review addresses the potential benefits of adopting RNAi methods to lower viral infectivity by targeting *PB1*. Here, controversies surrounding the assembly, functioning, and mutations involved in immune re-assortment of the *PB1* gene in influenza virus, particularly type A, are highlighted. RNAi strategies that reduce *PB1* expression and, thus, enhance the host immune response are compared.

Import and assembly of RNA polymerase

Influenza places a major socio-economic burden on countries [1,2,3]. According to a review in the *Journal of Antimicrobial Chemotherapy*, seasonal influenza accounts for a loss of nearly 10-15 billion dollars each year in the United States alone, due to absenteeism from work or school [1]. This results in higher indirect than direct costs due to lost earnings from absenteeism or loss of future earnings from death. In Canada, it is estimated that around 12,000 people are hospitalized due to influenza and as many as 3,500 deaths result annually [2].

There are three types of influenza- A, B, and C [3]. Influenza C cases are not as common as influenza A and B cases. Influenza A and B viruses are part of the *Orthomyxoviridae* family and are both negative-sense, single-stranded RNA viruses [2,3,22]. Although influenza types A and B are morphologically similar and possess similar mutation mechanisms, influenza B mutates and evolves at a slower rate compared to influenza A.

This review will focus on influenza A because the virus is more widespread and has a greater impact on society; the virus has been linked to major pandemics due to its greater genetic diversity and host range [1,2,3,22]. Influenza A targets humans and birds, whereas influenza B affects humans and seals. Human influenza A virus can re-assort with avian viruses, forming new combinations of surface proteins hemagglutinin (HA) and neuraminidase (NA). As a result, different types of strains circulate throughout the population annually, attenuating human immunity and increasing drug resistance. Whereas seasonal influenza is more acute, avian influenza (AI), an infectious viral disease of birds, can cross the species barrier and infect humans, with the potential for either high or low pathogenesis. In a highly pathogenic state, termed HPAI,

mortality is almost certain; in a low pathogenic state, termed LPAI, the disease is not as severe and mortality rates are not as high [2,3]. It is concerning to note that those infected with AI virus present no signs of illness initially.

In influenza A, there are 11 genes on eight pieces of RNA that encode 11 proteins [3,5,8]. Segments are assembled into ribonucleoprotein (RNP) complexes consisting of viral RNA (vRNA) enclosed by nucleoprotein (NP) and RNA-dependent RNA polymerase (RdRp) [8]. The RNA polymerase of the ribonucleoprotein (RNP) complex contains three subunits: polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic protein (PA) [5,6]. PB2 and PA contribute to the viral polymerase, but PB1 is most responsible for the formation of the structural backbone and for the catalytic activity of the RNA polymerase, increasing virulence [6]. In order for negative-sense, single-stranded segmented RNA viruses, like influenza A, to be transcribed and increase infectivity in the host, viral RNA-dependent RNA polymerase transcribes the RNA into usable complementary positive-sense strands and produces monocistronic mRNAs from the genome segments [3,5,6,7,22].

The exact interaction of the three proteins-PB1, PB2, and PA- and the timing of their import into the nucleus for polymerase assembly is disputed [7]. It is important to understand the assembly of these subunits, because subunit re-assortment and mutations in these components lead to increased viral infectivity [5,6].

Several possibilities for the temporal and spatial arrangement of the assembly of these three subunits have been suggested. By epitope tagging components PB2 and PA with GFP, Fodor *et al.*[7] observed outcomes consistent with three possibilities: 1) PB1, PB2, and PA all enter the nucleus, 2) PA alone enters the nucleus, 3) PA and PB2 form a complex that stabilizes and leads to the nuclear accumulation of PB1 later. Their data also suggested that PB2 was not needed for PB1 and PA nuclear accumulation. While GFP was used to visualize subunit assembly, only the PB2-PA complex was tested.

To substantiate the findings of Fodor *et al.* and test these possibilities, Huet *et al.* [5] investigated the associations and import of the three subunits using a technique to observe protein-protein interactions *in vivo*. The technique used, called fluorescence cross-correlation spectroscopy (FCCS), measures molecular interactions by looking at correlations in particle movement, which is observed by labeling two independent species with two fluorescent probes of different colors; after excitation, colors can be observed with a confocal microscope. The authors were able to apply FCCS to influenza polymerase experiments, in which they mutated the nuclear localization signal (NLS) of PB2 and disrupted polymerase formation [5] (Figure 1).

After the experiments of Fodor *et al.* [7] and Huet *et al.* [5], the import and assembly of PB1, PB2, and PA became clearer. It was acknowledged that PB1 and PA [5,7], and not PB2-PA or PB1-PB2 [8] as previously suggested, form a dimer and import into the nucleus, followed by the import of PB2 separately.

To support the findings of Fodor *et al.*, Huet *et al.* accounted for all combinations of double and triple co-expression missing from Fodor *et al.*'s study. However, the specific PB1-PA interaction was not researched further and the interaction outcomes of NLS mutations of PA or PB1 were not investigated. Direct examination of PB1-PA interaction and experimental ablation of PA and PB1

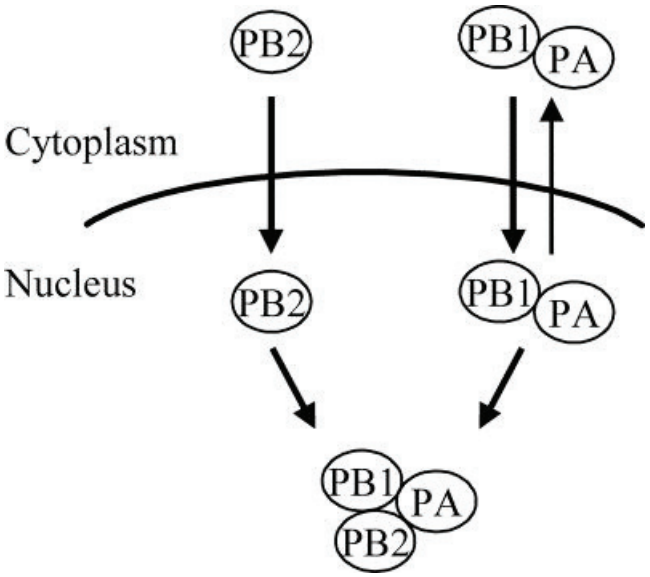

Figure 1: *Nuclear import and assembly of PB1, PB2, and PA.* The model proposes that PB2, once inside the nucleus, joins with PB1 and PA. PA enters the cytoplasm to join with PB1. PB1 and PA are therefore joined prior to entering the nucleus. A trimeric polymerase complex is formed. The three subunits PB1, PB2, and PA are part of the RNA polymerase, which plays a key role in regulating the replication cycle of the influenza virus, influencing infectivity. The mechanism of nuclear import and assembly of these three subunits is controversial. Having a more precise understanding of PB1 assembly will help connect viral replication processes to the host immune response, which will benefit studies employing RNAi methods [5].

nuclear localization signals are required, as they have not been performed to date. This is important for creating antiviral therapies, like vaccines or drugs, to lower the infectivity rate of influenza A. Information gained about PB1's precise structure and role, transcriptional regulation, and interactions with other subunits of the RNA polymerase [6,13] could provide support for the benefits of targeting *PB1* in future studies that employ RNAi methods.

Viral replication and the immune response

In the immune response to influenza A virus, the binding of virus-specific molecules to receptors, such as the membrane Toll like receptor-7 (TLR), induces a signaling cascade that eventually results in the release of interferons (IFN) [13, 14, 15, 16]. IFN-α and IFN-β are part of the host's defense response, which, when initiated, results in the activation of immune cells (macrophages) and antiviral genes called interferon stimulated genes (ISGs) that inhibit viral replication [13, 14]. More details are discussed in the section titled "*PB1* and RNA interference (RNAi)". However, viruses have developed mechanisms to oppose the host's IFN mediated defenses [14]. As Lee *et al.* [14] write, "viruses evolved to hijack the mechanisms of the cell for their own benefit- to efficiently replicate".

Examining the PB1 subunit is important because studies have demonstrated that different mutations in PB1 can alter replication and therefore affect IFN response, described below [13,14,15,16]. If polymerase function is inhibited, viral replication decreases, and the immune response can be promoted, to some degree. These events have important implications for the creation of antiviral therapies, such as vaccines, which could promote immunity. However, there are also multiple sites within PB1 that resist certain compounds

that inhibit viral polymerase function in virus-infected cells, which will be described below [6,13,17,18,19]. A clear understanding of the role of these sites can be used to create novel methods for targeting viral replication.

Ortigoza *et al.* [13] were the first to establish, through sequencing, that mutation of the conserved PB1 residue Y499 to histidine was responsible for conferring resistance to a compound, which was named ASN2. By screening molecules involved in inducing IFN, the authors found that ASN2 was an antiviral compound within virus-infected cells that could inhibit RNA-dependent polymerase function, while simultaneously promoting type 1 IFN (α/β) indirectly [13]. Importantly, it was shown that a link could be established between viral replication and the host immune response, that in the presence of the virus, the host recruits the immune response and viral replication can be inhibited. Although viruses have developed mechanisms to oppose IFN, drug therapies that indirectly promote IFN while reducing viral replication could utilize antiviral compounds, like ASN2. Another study found that Histidine 456, in PB1 could confer resistance to an anti-influenza compound that targets the *PB1* product, termed 367, by mutating histidine to proline [17]. After undergoing this mutation or alteration, viral mRNA transcription is not inhibited and thus influenza virus replication is not inhibited.

Minireplicon assays, powerful cell-based assays that measure influenza virus polymerase activity through reporter gene expression levels, were performed by Chu *et al.* [6]. Results from this assay suggest that polymerase activity is actually reduced by mutations in non-consensus sequences, not non-conserved regions, of PB1. Interestingly, avian virus PB1 mutations in conserved motifs show greater variability, among the four motifs, than in corresponding human virus PB1 [6]. It is unclear what implications this variability has on polymerase activity, whether this makes the virus better at replicating than the human virus or not. This study provides a different perspective on avian and human influenza in reducing polymerase activity: the human and avian PB1 proteins might mutate at different rates and function differently. In one study, Rolling *et al.* [18] used adaptation experiments to measure gain of virulence. It was found that an L208R mutation in PB1 increased polymerase activity and virulence [18]. Antiviral therapies could employ these data to alter PB1's biochemistry, thus decreasing replication and therefore reducing virulence.

Overall, these studies are stepping-stones in forming a complete picture of PB1 and its role in viral replication and infectivity to create antiviral therapies. Similar studies have been designed in influenza B and C [3,4], but more studies of PB1 in influenza A are needed to compare similar subunits in avian and human viruses.

PB1 and RNA interference (RNAi)

RNA interference (RNAi) is a natural cellular process that reduces gene expression by degrading mRNAs [4,20]. Once double stranded RNAs (dsRNAs) are generated by the Ribonuclease Dicer, the newly formed small interfering RNAs (siRNAs of 18-23 nucleotides) become incorporated into an RNA Induced Silencing Complex (RISC), where it associates with an Argonaute protein [20]. The siRNA is antisense to a particular mRNA target, and guides the Argonaute to target transcripts. Once bound to the target mRNA, the Argonaute has Ribonuclease activity that cleaves and degrades the mRNA, thus reducing the expression of a specific gene [20]. Three ways to use RNAi to regulate gene expression are summarized below.

First, one way to employ RNAi involves introducing siRNAs into cells in the laboratory, after which cells can activate the RNAi pathway to degrade expression of a specific gene [4,20]. In this technique, siRNA expression plasmids are delivered to the cell, where they further direct the synthesis of siRNAs that specifically target *PB1*. These siRNAs target *PB1* by degrading *PB1* transcripts [21], thus reducing the expression of the virus (and thus, the avian virus by degrading expression of the *PB1* gene). In contrast to the highly mutable HA or NA genes, *PB1* appears to be a suitable target for RNAi-based therapeutics due to its conserved regions [22, 23].

Second, the utility of *PB1* as a therapeutic target has been demonstrated by introducing short hairpin RNAs (shRNAs), a sequence of RNA in a hairpin shape, into cells [4,20,22]. shRNAs do not degrade as easily as siRNAs do [22]. Therefore, delivering shRNAs via an expression vector to cells is an advantageous method, despite the risk of safety concerns or toxicity when introducing expression vectors. The shRNA, once inside the cell, is transcribed in the nucleus by a certain promoter; the transcribed product mimics pri-microRNA, which is then processed by Drosha, eventually resulting in a cascade process, similar to that of siRNAs. shRNAs and their use are described in more detail below.

Third, shRNA and siRNA sequences can be modified and then delivered to the cell via expression plasmids [21,22]. Overall, RNAi has been a successful method in reducing *PB1* expression during influenza A viral infection of cells, usually of MDCK or HEK 293T cells [15,16,21,22]. The different ways to employ RNAi will be discussed in more detail below, highlighting recent studies that used these techniques.

A recent study by Stewart *et al.* [15] employed RNAi to target degradation of H5N1 *PB1* mRNAs. This type of degradation simultaneously activates type I IFN production, promoting an increase in the immune response. When siRNAs are delivered to the cell, immune cells are recruited; Toll-like receptor (TLR) 7 and TLR8 sense dsRNA. Upon activation of TLR7 in plasmacytoid dendritic cells, IFN-α production is induced and upon activation of TLR8 in monocytes and macrophages, tumor necrosis factor alpha (TNF-α) and Interleukin 12 (IL-12) production is induced [15,16]. In the study conducted by Stewart *et al.*, the authors modified siRNA by adding a 5-bp nucleoside sequence (5'-UFUFU-3') at the 5'-end of the siRNAs that were introduced into chicken and Vero cells [15]. Using this modification, the authors were able to maximize the immunostimulatory response by obtaining high levels, or what the authors called "rapid and enhanced induction", of IFN expression when their siRNA modifications were introduced into chicken cells. Importantly, the 5-bp nucleoside sequence could be tagged to *PB1*; using an anti-H5N1 avian influenza siRNA against the gene could effectively induce IFN (type I) at an earlier time point and in a greater amount, in the presence of the modification. This was shown to be the case in both *in vivo* and *in vitro* studies [15].

A study by Gantier *et al.* [16] also explored sequence and structural modifications of siRNAs. The authors designed their study by using modifications consisting of a 5'-triphosphate siRNA sequence and a miRNA-like nonpairing uridine-bulge structure in the passenger strand of siRNAs. The combination of using not only sequence modifications, but also structural modifications,

led to immunostimulatory activity (stimulation of the immune response), by recruitment of human TLR7 and TLR8, which plays a role in IFN pathway activation [16]. This is a useful outcome, because promoting the immune response may help strengthen the often hijacked immune system in those who contract influenza.

Using PB1-short hairpin RNA, or shRNA, appears to be a more advantageous technique than using siRNA because of longer lasting effects when introduced into the cell, as problems with degradation and toxicity are common in RNAi studies [22]. shRNA is cleaved to produce a 21-bp siRNA complementary to specific nucleotides on the viral RNA. This method is quite effective, as Madin-Darby Canine Kidney Epithelial (MDCK) cells infected with the avian virus and treated by this method exhibited significant inhibition of viral replication.

Notably, Li *et al.* [22] showed that not only could viral replication be inhibited in mice, but that survival rates of the infected mice could be increased as well after shRNA treatment. The authors cloned a PB1-shRNA oligonucleotide into a vector and delivered the resulting vector into MDCK cells. Introducing influenza virus to the MDCK cells transfected with the vector resulted in an inhibition of viral replication. In cell culture, virus titre was measured within 48 hours, and in mice, viral replication and survival rate were measured. The authors chose to target the *PB1* gene. Since degradation and toxicity upon delivery of the vector to cells is a potential disadvantage of RNAi, the authors were able to successfully manage these problems by using vector-only negative controls. The authors also accounted for off-target effects, in which the sequence of the small RNA can recognize another mRNA than the intended target, by using a specific design system and BLAST analysis for *PB1*-specific siRNA. As a result of delivering their vector, the authors found that survival rates in mice were near 100%, whereas without introducing the *PB1*-specific vector and introducing the influenza virus, survival rates of the mice dropped to near 50% (Figure 2). Based on their results, Li *et al.* were able to provide strong evidence that RNAi using shRNAs is a potent method and serves as a promising therapeutic.

These studies [21, 22] did not connect the use of shRNAs to immune response involving IFN. It would be worthwhile to show how such studies could be applied to the PB1 subunit in reducing avian and human influenza rates.

Although RNAi methods are powerful and have potential application for clinical therapies, some researchers still suggest that these methods contain too many disadvantages in toxicity and delivery to be useful [4,20]. However, this review has highlighted recent studies in which RNAi methods have proved to be highly successful in reducing viral replication and infectivity, thereby promoting the immune response. If RNAi methods targeting the *PB1* gene can be utilized successfully in humans affected by human or avian influenza A, then high infectivity and mortality rates could be reduced ; the strain on global health resources and productivity might be lessened [1,2,3].

Conclusion

Understanding the import, function, and structure of PB1 is necessary to connect its transcription and replication processes to host immune response. Further research concerning its exact

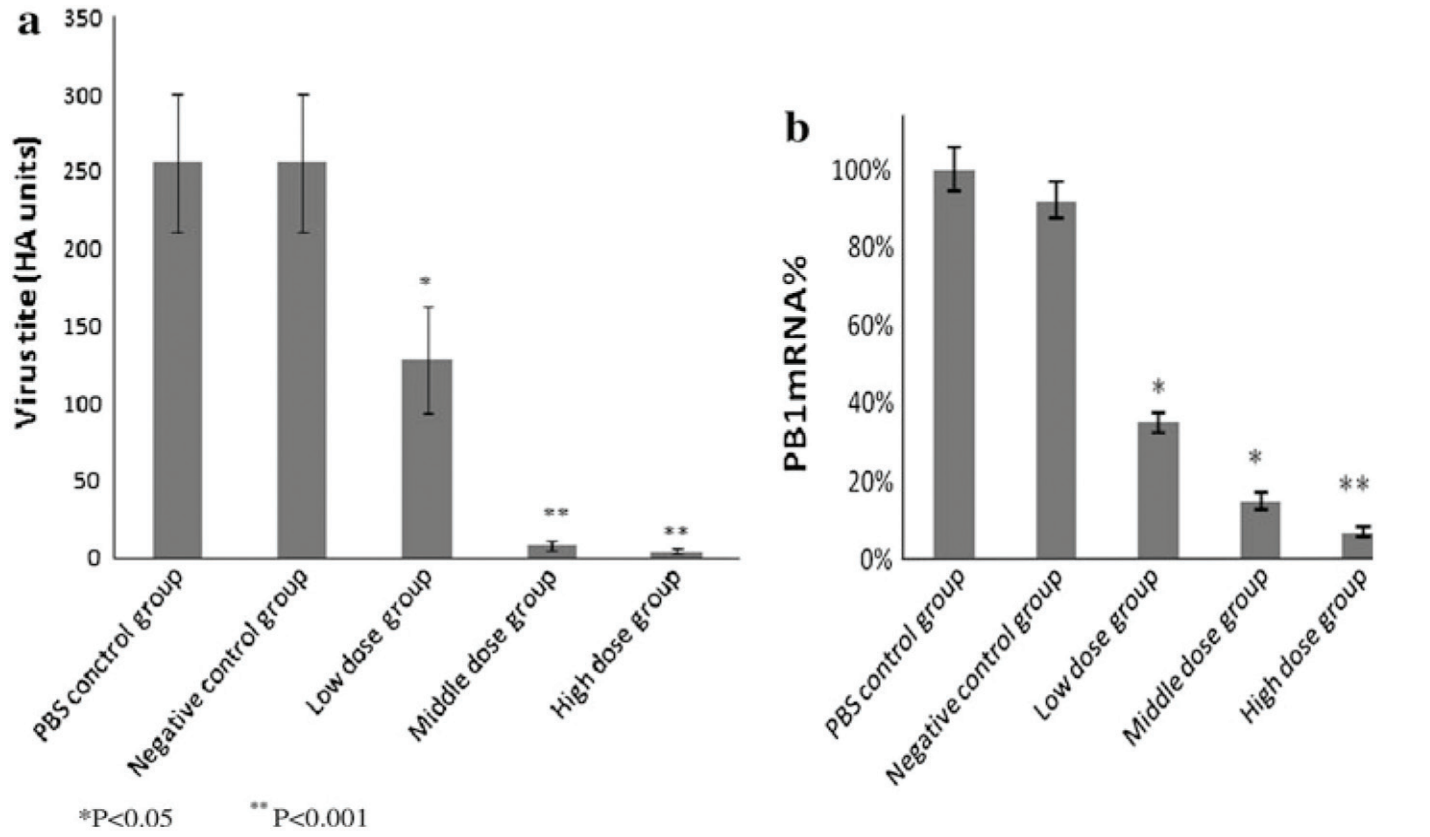

Figure 2: Inhibition of the influenza virus is possible using siRNA sequence modifications. a) Virus titre (amount present in circulation) decreases significantly when siRNA sequence modifications are introduced into the cell, as carried out in the study by Li *et al.* b) The recombinant plasmid used was statistically significant in decreasing PB1-mRNA levels. [22]

mutations at position 499 [13], connection to ASN2 and IFN [13], and dimer activity with PA [5] in all influenza types would be highly beneficial because virulence activity depends on re-assortment and mutation.

Based on the literature, *PB1* appears to be an excellent target due to its conserved regions [6,22,23]. Studies using RNAi methods, both shRNA and siRNA, have been successful in reducing *PB1* expression of the influenza A virus [15,16,21,22]. Experimenting with *PB1* in human viruses using RNAi methods can help understand and further reduce the replication and virulence of highly pathogenic avian influenza A viruses, like H5N1, for which little is known.

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JULS REVIEWS

The Feasibility of Surface Antigen-Based Vaccines for Variable RNA Viruses

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Abstract

Rapid replication rate and an error prone viral polymerase are the two factors that contribute to the remarkable virion heterogeneity in HIV-1 and HCV. Surface glycoprotein variability in these RNA viruses has thus been proposed as a mechanism for humoral immunity evasion. As the major antigenic targets, glycosylated surface proteins on HIV-1 and HCV have been investigated for use in protective inoculation. However, current literature reveals significant shortcomings in achieving cross-neutralizing protection via inoculation with these antigens, including conformational infidelity of expression systems, and the high titres of antibodies required to neutralize infection. This review summarizes the current challenges confronting effective surface-antigen mediated protection for variable RNA viruses, and explores the feasibility of non-neutralizing antibody-based protection in blunting the acute phase of infection for sufficient duration to enable cellular mediated immune clearance.

Introduction

The vulnerability of global populations to viral infection necessitates the advent of novel vaccines. Despite recent progress in antiviral therapies, prophylactic vaccines remain the gold standard for eradication and community wide disease prevention. Successful vaccination initiatives in the 20th century raised hopes for the continued elimination of disease-causing viruses. However, during the latter half of the 20th century this optimism has diminished as the repertoire of viruses susceptible to traditional vaccine design was exhausted.

Viral surface variability poses a unique problem. Error-prone polymerase mediated genomic mutations potentiate humoral immune evasion via continued generation of *de novo* viral variants unsusceptible to cross neutralization [1-5]. This population of virion variants within a host, termed quasi-species', is characterized by sequence conservation in non-structural proteins, and sequence divergence in epitopes immunologically visible to antibodies [6-8].

Differential genomic homology is explained by increased tolerability to mutations in structural relative to non-structural proteins (6, 9). Paradigmatic examples of variable surface protein immune evasion are Hepatitis C Virus (HCV) and Human Immunodeficiency Virus-1 (HIV-1).

Briefly, HCV and HIV-1 are enveloped RNA viruses whose cell entry is mediated by binding of viral surface glycoproteins to host cell receptors [10-12]. Subsequent endocytosis and viral uncoating initiate replication, which proceeds via direct translation of genomic RNA for HCV, and reverse transcription followed by integration into host genome for HIV-1 [10, 12]. Regardless of replication strategy, the implications of poor polymerase fidelity are immunologically conserved between HCV and HIV-1.

How Vaccines Work

The mechanism of vaccine-induced protection is not yet fully elucidated [13]. Beyond agreement on the necessity of lymphocytic memory, the nuanced partnership between T-cells and antibodies is unresolved. Particularly challenging is the understanding of how inoculum induced immunity might differ from natural host clearance. Robust cellular responses associated with spontaneous resolution of infection complicate the understanding of the importance of antibodies in primary infection [14]. Equally unclear is whether the protective effect of vaccines is mediated by antibody neutralization, or if antibodies predominantly act to augment T-cell response by sufficiently attenuating early infection.

Currently, all vaccines involve host exposure to antigens immunologically visible to B-cell receptors [13, 15]. Inoculum candidates thus include whole-killed virus, surface proteins, and attenuated virus, which will additionally induce cellular immunity but involves the risk of wildtype reversion [13, 16]. The common prerequisite of antibody paratope specificity to virion surface epitopes is therefore problematic when confronting surface mutating viral species. Briefly, if the antigen recognizing domain of the antibody confronts continually changing surface antigens, it will be unable to bind and neutralize the virion.

Considering surface stable viruses, the theory of vaccination is simple [13, 17, 18]. Host exposure to pathogen surface antigens stimulates clonal expansion of B-lymphocytes expressing specifically reactive immunoglobulins and their differentiation into plasma cells [18]. Pathogen-specific protection is then conferred via plasma cell mediated maintenance of high antibody titres, or memory B-cell stimulation upon renewed antigen

exposure [19]. Vaccine efficacy is therefore determined by both duration and neutralization capacity of viral antigen-specific immunoglobulins [20].

However, decreasing titres of neutralizing antibodies (nAb) following vaccination indicate protection is augmented by other immunological responses [13, 20]. Reactivation of memory B cells and activated cytotoxic T lymphocytes are the prime candidates for the sustained immunity despite diminished Ab titres [13, 18, 19]. Importantly, CD8+ T Cell effected viral clearance may be inadequate in isolation from the humoral immunity [21]. From this nuanced interplay of immunological responses emerges a working model of vaccine-based protection. Pre-exposure to surface antigens stimulates a humoral immune response that sustains efficacy of nAb via either long-lived plasma cells, or memory cells expressing B-Cell Receptors with paratopes specific to viral structural epitopes [13]. The associated immunoglobulins then attenuate early infection either by directly neutralizing virions, or augmenting a *de novo* cytotoxic response. A precise model of protection is complicated by host polymorphisms, and the enormous heterogeneity of viruses.

Can Antibodies Neutralize HCV?

The neutralizing efficacy of HCV-specific antibodies has been difficult to establish. Complicating our understanding, aside from surface antigen epitopic variability, is the *in vivo* association of viral particles with host lipoproteins that structurally conceal cell-entry related epitopes on the HCV surface glycoprotein E2 [22].

Antibodies for Hepatitis C Virus are neutralizing and cross-neutralizing [22-25]. Immunoglobulins isolated from the serum of chronically infected individuals demonstrate both quasi-species specific, and trans-genotypic neutralization [22, 25, 26]. These findings indicate virion susceptibility to variable and conserved epitope reactive paratopes.

Literature on antibody-mediated HCV control has identified both linear and conformational epitope targets for neutralization on E2 [22]. Importantly, presence of nAb specific to conserved epitopes does not tightly correlate with viral clearance [12, 27, 28]. This results from the low immunogenicity of conserved relative to variable epitopes on HCV E2 (Figure 1.) Humoral immunity evasion is thus partially mediated by immuno-dominance (increased immunogenicity) of variable epitopes, and their consequent antigenic masking of more conserved targets for neutralization [22]. However, though conserved epitope-specific antibodies are insufficient for viral control alone, their efficacy in the presence of quasi-species specific antibodies and cellular immune factors indicates viral vulnerability to broadly reactive immunoglobulins.

Inoculation with HCV glycoprotein heterodimer E1/E2, the viral surface proteins that mediate host entry, results in homologous and heterologous protection in chimps, respectively [28]. We acknowledge the limits of protection indicated by the host susceptibility to autologous reinfection (re-challenge with the same strain) [29]. Host factors underlying these differential responses are not fully elucidated, but it is established that re-infection in chimpanzees is attenuated and does not cause liver disease [28]. Thus, evidence of re-infection post-acute resolution does not negate the protective effect of E2 specific antibodies reported *in vitro*, *in vivo*, and clinically.

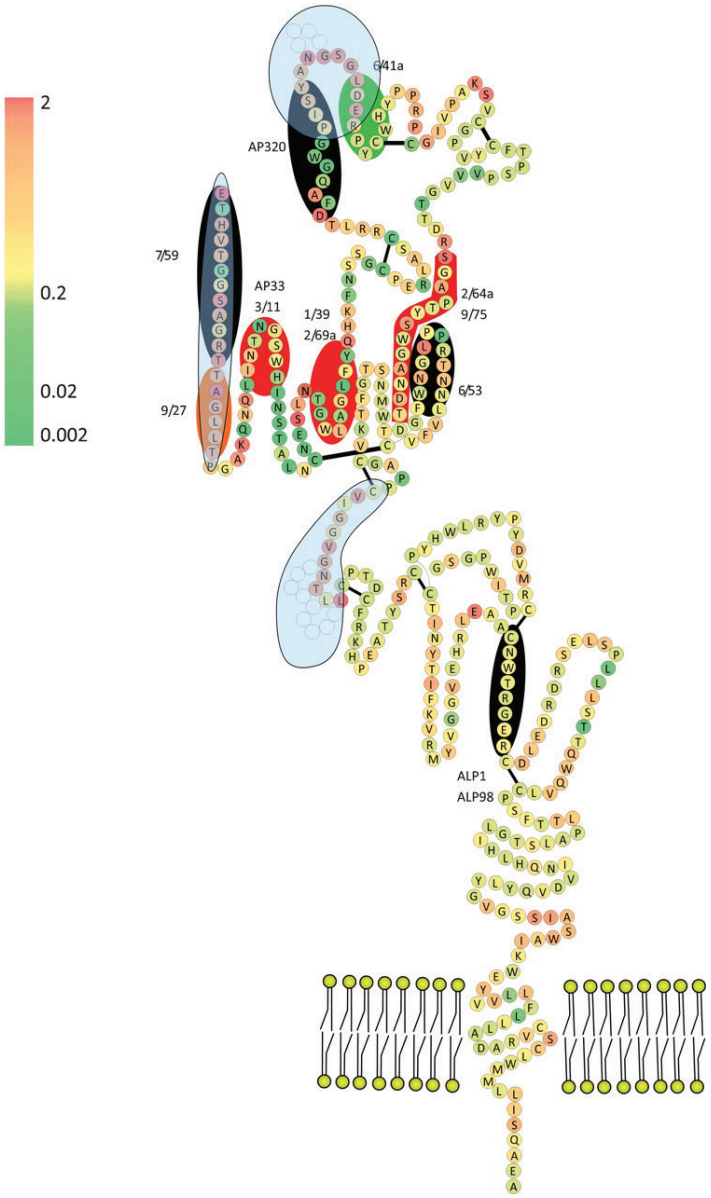

Figure 1: Proposed structure of HCV E2 GP color denoted by amino acid variability, with red indicating high variability (22). Putative hyper-variable regions denoted by translucent blue.

Recently a team in Edmonton demonstrated gpE1/E2 inoculum induced antibody neutralization against heterologous HCV genotype1a challenge [24]. Surprisingly, in one vaccine, inoculation with gpE1/E2 from a single viral isolate, and produced in mammalian cells, elicited a cross-neutralizing response against subtypes representing all 7 HCV genotypes. This study supports the concept that humoral mediated protection from quasi-species does not rely on pre-exposure to the specific variant of challenge [24]. *In vivo* surface antigen-based vaccine studies have yielded similar promising results. Chimpanzees vaccinated with HCV 1a gpE1-E2 were protected from chronic infection in over 80% of homologous challenges [28]. Strongest responders developed sterilizing immunity via gpE2 specific CD81 interaction interfering antibodies. Crucially, the same experiment reported robust (>85%) reduction in chronicity in vaccinated heterologously challenged chimpanzees [28]. Though sterilizing immunity was not observed in any of the heterologously challenged animals, it is not clear whether this robust protection is a

reasonable, or important, objective. Prevention of chronic infection is a tenable outcome of surface glycoprotein based HCV vaccines.

Difficulties with Vaccine Induced HIV-1 Specific nAb

Investigation of HIV-1 Env glycoproteins have not yielded viable vaccine candidates. Virion susceptibility to neutralization is diminished by heavy surface protein glycosylation and the physical concealment of epitopes by poorly immunogenic glycan shields [30, 31].

Inducing HIV-1 surface protein (gp120) nAb by vaccination confronts challenges similar to HCV [10, 32]. Poor reverse transcriptase fidelity potentiates novel surface protein variants unsusceptible to immunoglobulins generated by a prior event of clonal expansion [31, 33]. Quasi-species' coupled with the high immunogenicity of gp120 ectodomain variable loops ensures successful humoral evasion [30]. Despite host immune inadequacy, mAbs with broadly reactive neutralization have been observed [32, 34]. Unfortunately, the epitopes recognized by these Abs have proven difficult to express for inoculum use [10, 11]. Non-continuous, conformational epitopes constitute the predominant targets for neutralization [34, 35]. One notable exception is the linear epitope on the membrane-proximal ectodomain of gp41 recognized by mAb 2F5 [32]. Efforts in vaccine generated 2F5-like nAb have failed despite *in vitro* expression of the targeted peptide [32].

This suggests that *in situ* conditions of the HIV-1 virion surface proteins are not currently replicable by existing protein expression systems. Difficulties involved in HIV-1 vaccine design thus include the quasi species' problem, the predominance of conformational dependent neutralization targets, and the inability of *in vitro* expression systems to generate epitopes antigenically faithful to the *in situ* virion.

Future Vaccine Strategies

The failure of conventionally designed vaccines in preventing infection by surface variable viruses has prompted investigation of novel strategies. Both the stimulating antigens and delivery mechanisms have been re-analyzed to identify ways of overcoming viral defenses. The current shortcomings and future potential of these approaches will be briefly examined.

Virion structural variability has prompted vaccinologists to target conserved epitopes on the ectodomains of surface expressed glycoproteins [36, 37]. Two optimization approaches have been adopted for this strategy. The first involves elucidation of the biochemical interactions between virion epitopes and host cell entry factors. Precise determination of the residues involved in receptor recognition coupled with corresponding nucleotide sequence alignment enables expression of inoculum intended peptides [22]. The second strategy, reverse vaccinology, analyzes the paratopes of broadly neutralizing mAb to determine their reactive epitopes [38]. Amino acid characterization of these epitopes, followed by BLAST alignment, are used to reveal their coding genomic sequence. Amplification of target sequences for plasmid construction facilitates expression of epitopes specifically reactive to previously identified mAb [13].

An alternative to direct inoculation with humoral stimulating surface antigens is DNA vaccination. This approach involves transfecting host-cells with plasmids encoding viral antigens [39]. *In vivo* expression of viral proteins increases conformational fidelity, thus enhancing efficacy of immunoglobulin:antigen recognition upon infection [40].

Recognizing the inherent limitations of surface glycoprotein based vaccines for variable viruses, researchers have begun investigating the potential for non-structural protein based, or T cell, vaccines. The rationale for this approach derives from viral intolerance of mutations in key replicating enzymes [6]. Accordingly, despite high genome wide polymerase infidelity, non-structural protein mutants will be less viable on average than surface protein mutants (quasi-species) [41]. The *in situ* effect of intolerance will be increased with conservation in genomic regions encoding replication-involved enzymes. T cell vaccines, therefore, have the advantage of introducing antigens subject to high selective pressure *against* variation. Furthermore, robust CD8+ responses associated with viral clearance support the use of T cell mediated pre-exposure protection [42, 43].

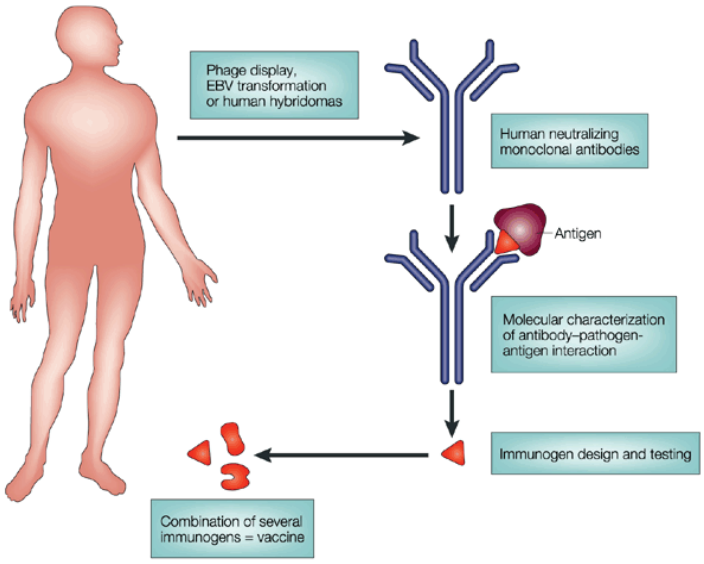

Figure 2: Reverse Vaccinology Using Host Derived nAB (13). Innoculating antigens are reverse designed from AB.

Concluding Remarks

Sterilizing immunity is not a tenable objective of variable virus vaccine design. The possibility of escape mutants in any vaccine strategy will be a persistent challenge. However, the potential for chronic preventing vaccines for non-immunocompromised subjects is not diminished by the untenability of sterilizing vaccines [13]. Prompting host-immune response pre-exposure could sufficiently attenuate early infection to enable viral clearance. Crucially, if Abs are to blunt the infection, robust humoral responses must be mounted before viral titres and virion variability peak [15, 17]. Evolution of defenses against nAb suggests the presence of ongoing selective pressure on HCV and HIV-1 by immunoglobulins. Identification of cross-reactive mAb raises hopes for future protective vaccines.

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JULS REVIEWS

The efficacy of interventions for ADHD in children and adolescents: A critical review

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Abstract

Attention-deficit/hyperactivity disorder (ADHD) is a heterogeneous condition determined in part by genetics, environmental factors and comorbid mental illnesses [1]. There is a need for multiple interventions in order to address the heterogeneity of ADHD and improve intervention effectiveness across individuals. A review of the literature indicates that interventions for ADHD can be compared and contrasted according to multiple criteria including (but not limited to): clinical utility, cost, side effects, long term and short term prognosis, developmental considerations and empirical evidence. This paper critically summarizes the advantages and disadvantages of stimulant medication, psychodynamic play therapy and executive functioning remediation training as common interventions for ADHD. Each of these therapies has distinct characteristics which can be tailored to the needs of the individual and have the potential to be combined to maximize child improvement (Table 1).

Introduction

ADHD is characterized by age inappropriate symptoms of inattention, hyperactivity and impulsivity which interfere with everyday functioning; the symptoms must appear prior to age 7 and persist for at least 6 months [2]. There are three different subtypes of ADHD: Inattentive, Hyperactive-Impulsive, and Combined Type. The disease has about 7% prevalence amongst children and adolescents in North America and about 5% worldwide [3, 4]. Males outweigh females in ADHD diagnosis by a ratio of about 2.5:1 in both children and adult groups based on empirical data [5]. The ratio rises to 6:1 in clinical samples, which may reflect a gender bias in diagnosis: males are referred more often because they are more likely to display inappropriate externalizing symptoms of ADHD than females [6].

There have been many explanations for the causes of ADHD. Some studies posit that ADHD is a trait left over from human ancestors who were hunters [7] while other theories link ADHD to high intake of sugar, vitamin deficiency, harsh parenting and other environmental issues [8]. Even though there is strong public belief in some of these claims, research findings have been inconclusive. Indeed, there is established evidence of malnutrition and environmental stress in a subset of children with ADHD [9] and there have been some studies showing symptom improvement in those following a special diet compared to a control group [10]. However, these studies have been mostly correlational, lacked appropriate levels of controls, or have not been robustly replicated [8]. Current research indicates that ADHD is a neurodevelopmental disorder arising from complex interplay between genes and environment with no single straightforward explanation [11]. The risk of ADHD development is as high as 8-fold in children with siblings with the disorder. As well, adoption studies have found rates 3 times higher in biological parents than adoptive parents of children with ADHD [12].

Currently, the first line of treatment for ADHD is stimulant medications such as Adderall and Ritalin [13]. Since stimulants do not treat the underlying cause of the disease, it is acknowledged that individuals may also benefit from interventions such as psychodynamic play therapy and executive functioning remediation training. There are several reasons why non-stimulant interventions are worth considering. Firstly, some studies have shown that stimulant medications can slow growth in young children [14]. In addition, the effectiveness of stimulants decreases with age in some children [15] and about 20% of children do not respond to medications or experience considerable side effects such as sleep disturbances, psychosis and loss of appetite [16]. Finally, the risk of misdiagnosis is high, meaning that there is a significant possibility of misprescription [17].

This paper critically reviews the current research on the advantages and disadvantages of these treatments and compares and contrasts each relative to the other in terms of clinical utility, cost, experimental evidence, side effects, long term and short term improvement, and developmental considerations (See Table 1). The reader should note that controversial treatments with weak experimental support [18] including dietary modifications, herbal medicines, and homeopathy are not considered in this review.

Table 1. Summary of the criteria used to compare and contrast stimulant medications, psychodynamic play therapy and executive functioning remediation training.

	Treatment type	Cost	Experimental evidence	Side effects	Effectiveness
Stimulants	Pharmaceutic	Low	Strong	Yes	Short term
Play therapy	Psychodynamic	High	Insufficient	No	Long term
Remediation Therapy	Brain training	Low	Growing	No	Long term

Stimulant medication

In 1937, Dr. Charles Bradley first noted the effectiveness of stimulants in the treatment of ADHD. While working with children with major difficulties in learning and behavior, he found that Benzedrine sulfate, an amphetamine salt, led to significant improvement [19]. Children showed an enhanced interest in school work, were more alert, and had diminished distractibility and behavioral disturbances [20]. Later work has shown the effectiveness of stimulant medications in alleviating hyperactivity and inattention symptoms to be robust. About 80% of children who take stimulants report a better ability to control impulses, stay on task and complete work in school [16]. Stimulants increase the level of the neurotransmitters dopamine and norepinephrine in the striatum and prefrontal cortex, causing improvement in executive functions such as self-control, concentration, and socially appropriate behavior. However these regions are part of the brain reward circuit, causing stimulants to have a high abuse potential [21].

There have been many studies in support of the efficacy of stimulants [22-25].The Multimodal Treatment Study of Children with ADHD (MTA Study) has been a landmark in this regard [26]. 579 children (7-10 years old) with ADHD Combined Type were randomly assigned to either (1) the medication management group (2) community care (3) behavioral treatment (4) behavioral treatment or the medication management (combined group). Children in the medication group were given Ritalin at the optimal dose and were carefully monitored. The community care group received psychotherapy and/or medication, but the medication was either different from stimulants or was given at lower than optimal doses. In the behavioral treatment group, teacher and parental support programs were also provided to children (Adapted from work of Barkely, Forehand and MacMahon) [27, 28]. The treatment continued for 14 months, after which these groups were compared based on parent and teacher report forms of hyperactivity and inattentiveness symptoms (Swanson JM School-based Assessments and Interventions for ADD Students, SNAP), academic performance (Wechsler Individual Achievement Test, WIAT), grade point average (GPA) on a 4-point scale, and social skills (Multidimensional Anxiety Scale for Children, MASC). All groups showed marked improvement in their scores and symptoms, but the effect was most increased in the medication groups.

The MTA follow up studies failed to show any significant difference based on randomized treatment group assignment, suggesting that children had benefitted from all the treatment types to an almost equal extent [29-31]. There have been several suggestions as to why the superior advantage of medication diminished in the follow-up studies. Breggin [32] argued that the MTA study lacked a control group, and the placebo effect has not been addressed. In addition, the results were based heavily on reports of teachers and parents, who were not blind to the experiment. While this is true, the study did include measures of academic ability (WIAT and GPA) and revealed that academic performance is markedly lower in children with ADHD compared with healthy controls. Furthermore, recent studies have found that stimulants can even alter brain structural abnormalities when taken for a long time, supporting that stimulants can induce brain plasticity [33]. Finally, after 14 months of direct supervision, children were left on their own, and MTA study became observational in nature. Therefore, it is possible that some children did not continue to take their medication as regularly as before [29]. Overall, it seems that stimulants provide symptom relief, but whether pharmacological treatments can yield positive long-term outcomes in the majority of individuals remains unknown. Most likely, the drug effectiveness depends on multiple factors such as the severity and types of symptoms, comorbid mental disorders, and age of onset.

Psychodynamic play therapy

Psychodynamic play focuses on underlying causes of maladaptive behavior by encouraging the child to play games. Psychoanalysts can then interpret the child's behaviour and help the child cope with the symptoms. The rationale is that ADHD symptoms can be both a cause and a consequence of behavioral disturbances. Therefore, a treatment which focuses on the behavioural symptoms can lead to improvement in brain functioning [34]. Further, play therapy is believed to provide a longer and potentially more stable improvement in ADHD symptoms. In a qualitative study [35], Cione and coworkers described a case study with a 6-year-old boy with ADHD, who had difficulty in school including marked distractibility and extreme mood swings. When referred for psychoanalytic therapy, the therapist realized that the symptoms stemmed from his jealousy for his brother, and he would project out his "badness" in school by acting aggressively toward others or being unable to concentrate. Addressing the underlying causes of aggressive behavior led to significant improvement. The authors proposed that this was because the psychodynamic therapy had provided a safe holding environment that was supportive and nurturing. The importance of supportive environments was also noted in the MTA studies wherein the subset of children with better peer and parental support showed a better prognosis [15]. In another longitudinal randomized study [36], preschool children (3-6 years old) were divided into 2 groups, receiving either psychoanalysis therapy or no treatment. ADHD diagnosis was defined by the Child Behaviour Checklist (CBCL4-18), Conner's Teacher's Rating Form (CTRF) and Conner's Parent's Ratings Form (CPRF). The experimental group received intense psychoanalytically based supervision in kindergarten, psychoanalysis educational programs, and individual and parental counseling for 2 years. Following the intervention, children showed significant improvement in aggressive and anxious behaviours compared with the control

group. These benefits persisted into the following school years, suggesting that the intervention and prevention psychoanalysis therapy had positively improved social functioning.

While there is evidence that psychoanalytic therapies can lead to marked prognosis in children with ADHD, these findings have not always been replicated for several reasons. Firstly, the psychoanalytic protocols have not been strictly followed in different studies given the unstructured nature of the therapy [37]. Additionally, the studies supporting psychoanalytic approach have used multiple measures to evaluate a reduction in symptoms, and this variation could have translated into inconsistencies [38]. Finally, it will be interesting to test the efficacy of psychodynamic approaches in older children since developmental stage and social responsibilities can influence the symptomology and prognosis of ADHD [29]. For instance, being inattentive has been shown to negatively correlate with the emergent literacy skills in preschool children while there is no link between hyperactivity/impulsivity and reading-related skills [39]. Despite all these limitations, there seems to be a need for psychosocial interventions at least as a complement to current medications since stimulants target the symptoms only and do not address the underlying causes of abnormal behavior [34].

Executive functioning remediation training

Non-stimulant medication interventions are of great importance in the treatment of ADHD because as many as 80% of children with ADHD also receive another diagnosis; these children may suffer from conduct disorder (CD), oppositional defiant disorder (ODD), learning disorders, anxiety and depression [40]. In cases of ADHD with comorbid mood disorders, for instance, stimulants are not effective in dealing with symptoms of depression and pharmacological treatments for depression do not alleviate ADHD symptoms [41,42]. Therefore, there exists a need to address the common etiology of the symptoms in comorbid cases. In fact, executive functions such as concentration, working memory and response inhibition are impaired in both ADHD and depression, and executive function training, in theory, should lead to considerable prognosis as a result [43, 44].

In a recent double-blinded randomized study by Oord and colleagues [44], 40 children aged 8-12 years with a diagnosis of ADHD were assigned to either the executive functioning (EF) group, EF and Ritalin (combined group), or a waitlist (control group). Children in the EF group played a computer game for a total number of 25 times in 5 weeks. Each playing session involved training of working memory (WM), inhibition control and cognitive flexibility. After 5 weeks, children in treatment groups showed significant improvement in executive function as measured by their pretest and posttest scores of Behavior Rating Inventory of Executive Functioning (BRIEF), a test with good test retest reliability [45]. Parental reports also showed a marked reduction in inattentiveness, hyperactivity and impulsivity. Parents were not blind to the experimental procedure and arguably, they could have introduced bias. However, teachers had no knowledge of assigned treatment groups, and their ratings confirmed parental ratings. Finally, a 9-week follow-up with participants replicated the original findings indicating long term efficacy of EF remediation training.

Children in EF group improved to the same extent as those in the combined group, suggesting that the stimulants did not confer an additional benefit. The fact that children enjoy playing games can and should be used to design treatments that are appealing to children given that EF training has no side effects and more importantly, it leads to improvement in cognitive functioning, which can definitely improve the quality of life for children with ADHD. In future studies, it will be interesting to figure out which subsets of EF are most effectively trained using computer games [46]. For instance, a recent meta-analysis of 25 studies found that while short term memory training resulted in improvements, the benefits from combined working memory and executive functioning training were not significant [47]. In addition, it will be informative to conduct brain-imaging studies in order to check whether the treatment can change brain structural abnormalities. This will provide objective evidence for the long-term efficacy of EF training.

Conclusion

Although it is known that ADHD is a neurodevelopmental disorder and is greatly affected by genetic factors [11], the heterogeneity of the disorder necessitates a variety of interventions. For instance, stimulants help normalize neurotransmitter and structural abnormalities [21] while computerized EF training can improve working memory and executive functioning [44]; psychodynamic approaches can help with social problems the child may experience in every-day functioning [35]. We should keep in mind that each treatment possesses advantages as well as limitations, and the evidence suggests that treatment effectiveness is dependent on developmental stage. For instance, psychodynamic play therapy can result in improvement in preschool children, but the evidence remains scarce for its efficacy in older children [48].

Available stimulants are effective in the short run, inexpensive, improve every day functioning by alleviation of core symptoms, and can mediate brain plasticity. However, they are not as effective in the long term and slow the growth rate in some children [15]. Psychodynamic therapy is patient-centered and takes into account individual differences, the process of treatment is flexible, and the therapy aims to address social factors that can exacerbate core symptoms. Nonetheless, this approach has not been widely accepted in the medical community, is expensive and has low interrater reliability [34]. EF remediation training, on the other hand, is inexpensive, appealing to children and effective in the long run. However, it is not clear whether the efficacy of computerized games can be replicated in most cases due to low statistical power of studies in this regard [49]. One day, it may be possible to administer distinct treatments for multiple variations of ADHD, but for now, all effective treatments should be considered in order to maximize the child's gains and to minimize the complications of ADHD.

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JULS REVIEWS

Nonlinear Dynamical Analysis of the Electroencephalogram: a review of theory and applications

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Abstract

Electroencephalography has long been established as a suitable technique for studying spatiotemporal dynamics of brain electrical activity. Traditionally, it has relied on classic statistical methods to interpret the electrophysiological signal, and it has generally focused on the linear features of the electroencephalogram (EEG). However, these methods were largely devised for the analysis of oscillatory behaviour, an approach that omits subtle but quantifiable dynamics that exist within the non-oscillatory EEG. The exploration of the non-oscillatory signal by the recent application of nonlinear dynamical systems theory to EEG analysis has uncovered interesting new findings with respect to the dynamics of the EEG in pathological conditions. Emerging trends suggest that the normal EEG signal has low synchrony and fractal features with high complexity. In pathological states such as epilepsy, the EEG exhibits hypersynchrony with diminished fractal features. Conversely, degenerative disorders such as Alzheimer's disease produce a disordered EEG of low synchrony and low complexity. This paper provides a brief overview of nonlinear dynamical analytic techniques and summarizes recent advances in their application to electrophysiology while also considering their limitations.

Introduction

The field of electroencephalography has seen a resurgence of interest in recent years due to promising new applications of dynamical systems theory in neurophysiology [1,2]. Traditional approaches to the analysis of EEG signals have relied on statistical measures such as coherence, cross-correlation and spectral analysis. While these methods are well-suited for the study of regular, ordered patterns such as oscillations and synchrony, they overlook a large component of information that lies within the seemingly stochastic fluctuations of the EEG signal. This focus on ordered phenomena is derived from a reductionist approach in physical sciences where complex, self-organizing phenomena such as those found in living systems are rarely considered. Spectral analysis may accurately be applied to temperature oscillations where each contributing member of the statistical whole, such as particles in contained matter, has random Brownian properties. However, the limitations of these methods become evident when applied to complex systems such as trade markets or living organisms, where the behaviour of each elemental unit displays agency and does not behave randomly. Such systems, comprised of numerous dynamically interacting components, have proven difficult to predict by classical spectral methods. Recent theories of brain function have proposed that the immense network of neurons, organized into vast communicating hubs, constitute just such an unpredictable, nonlinear dynamical system [3,4]. Several advances in mathematical theory as well as new techniques in time series analysis have now made it possible to apply nonlinear dynamical systems analysis to electrophysiological signals. The following description of this application of mathematical theory is largely based on the theoretical and technical protocol given in Kantz and Schreiber [5].

Nonlinear Dynamical Systems Theory

Dynamical systems can be characterized according to their propensity to incorporate and sustain external disturbances. A linear or an oscillating system, for example, may absorb a disturbance but will quickly return to its equilibrium state. In chaotic systems, a small disturbance may drastically shift the course of the system's evolution and permanently affect the succession of future states. The evolution of these systems is determined by a set of laws or differential equations that assume different values as the system evolves. In order to visualize the trajectory that these systems follow over time, researchers make use of phase space plots where one point represents the values of all the variables of that system in a particular point in time (the state of the system). The line that connects these points as the system moves from state to state is called the trajectory of the dynamical system.

A crucial feature of some dynamical systems is that the trajectory eventually converges into a subset of the total phase space which is termed the "attractor" (Figure 1). In a linear dissipative system, such a damped pendulum, the values of all the variables that describe the motion return to a fixed state after the initial perturbation. The trajectory in the phase space plot depicts a spiral that converges to a point attractor corresponding to the resting state

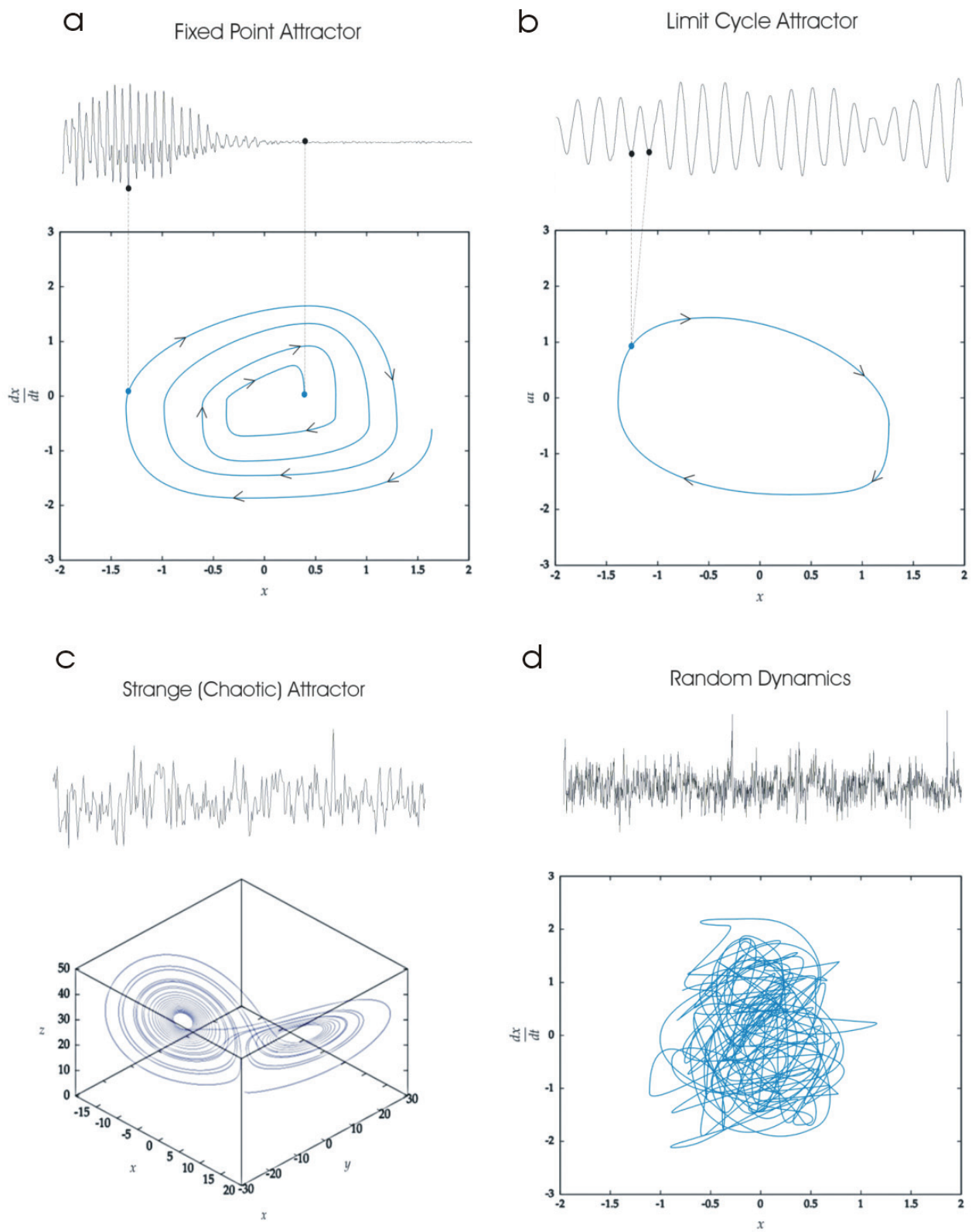

Figure 1: A diagrammatic illustration of different EEG signals and their reconstructed attractors. In panel A, the system signal comes to a steady, non-oscillatory state after some rhythmic disturbance. Plotted in phase space, these dampened oscillations are depicted by a spiral trajectory as the values of the EEG converge to a steady state represented by a point in the phase plot. In oscillatory systems (panel B), the trajectory settles to a limit cycle attractor that represents the rhythmic increase and decrease in the values of the EEG signal. In the chaotic EEG (panel C), which is not easily discriminated from a random signal, the trajectory never repeats itself although the system converges onto an attractor that has a complex, fractal structure. The attractor of the random signal, by comparison, is not characterized by any clear, structured geometry (panel D). Extracting the underlying attractor of an EEG signal can provide subtle information about the dynamical state of a recording site. A highly synchronous EEG with low complexity is indicative of epileptiform activity whereas low synchronicity with low complexity characterizes the EEG in dementia. For the purposes of concision and clarity, the EEG traces as well as the corresponding phase plots were created in CorelDraw9. For examples of actual phase plot reconstruction, see references [2, 3, 5, 18].

values. In periodic motion, the system repeatedly deviates from and returns to its starting point, tracing out a looped trajectory in phase space called the limit cycle attractor. For example, the attractor of a system characterized by perfect sinusoidal oscillations forms a circle. In a random system, the trajectory has a low degree of order and does not converge onto any geometrical shape because the collective values of the system do not frequently repeat.

A chaotic system has the unusual property of converging to an attractor but never repeating a previous trajectory as it courses from state to state. Chaotic attractors are also characterized by a unique geometric structure produced by the unique trajectory which is not intrinsically ordered like an oscillation but is also not fully random. While the loop is a 2-dimensional structure and the point has a dimension of zero, the chaotic attractor is characterized by a fractional dimension whose value lies between integers. This property, first described by Benoit Mandelbrot [6], is due to the slightly irregular features contained within a chaotic structure. In Figure 1, for example, the chaotic attractor resembles a 2-dimensional loop; however, the non-repeating but self-similar trajectory that comprises it gives it a dimension that is closer to 3. Because the values never repeat, the trajectory of the curve contains randomness. However, the trajectory appears contained within loops that are similar to the whole structure. By determining the dimension and the self-similarity of the attractor, a researcher can thus uncover possible chaotic dynamics that are overlooked by spectral analytic techniques designed to analyze only limit cycle dynamics. (For a more detailed review of fractal geometry in medical signals, see the recent publication by Lopes [7]). Another crucial property of chaotic attractors is their sensitive dependence on initial conditions. Infinitesimally small differences in the initial parameters of two trajectories in the same chaotic system can produce vastly divergent paths; small causes can have disproportionately large consequences. This property makes chaotic systems very difficult to predict beyond short periods of time. These systems are paradoxically both deterministic, as described by equations, but also unpredictable due to their sensitivity to initial conditions [8].

The rate at which trajectories with different initial conditions diverge can be calculated by a measure called the Lyapunov exponent. A positive exponential divergence indicates that a small difference in the initial values of the two systems produces disproportionately large differences in their trajectories. This sensitivity to initial conditions is indicative of chaotic dynamics. A system where the sum of all Lyapunov exponents is negative, on the other hand, indicates convergence to a point or to a limit cycle attractor. Another interesting dynamical measure that relates to the Lyapunov exponent is information entropy, which is defined as the rate of information loss over time. Mathematically, the entropy of a system equals the sum of all Lyapunov exponents [5]. Thus, a conservative dynamical system that does not dispel energy and does not lose information has a zero sum of Lyapunov exponents and zero entropy. Dissipative systems, on the other hand, experience energy (information) loss over time and have entropy that is equal to the sum of all Lyapunov exponents of that system. Chaotic systems are characterized by positive entropy and a positive Lyapunov exponent, reflecting their sensitivity on initial conditions. A nonlinear dynamical system can also experience perturbations that can cause the trajectory to shift from one attractor to another. This property, termed multistability, allows for smooth transitions between attractors. With sufficiently large perturbations, a dynamical system can undergo a qualitative transformation (bifurcation) whereby an old attractor disappears and a new attractor with novel properties is created [2].

Nonlinear Analytical Techniques in EEG Studies

The early application of these concepts to EEG analysis was particularly difficult as techniques for extracting the underlying dynamics of an EEG signal were scarce. However, recently-developed methods now allow researchers to confidently approximate the geometry and dynamical properties of the underlying attractor [9]. It is possible to express the fluctuations of the EEG signal as a dynamical trajectory by plotting the values of each data point as vectors in phase space. Mathematical proofs have shown that this reconstructed trajectory displays the same dimension and Lyapunov exponents as the true trajectory of that system [10] . The progression of this sequence of vectors is taken to be equivalent to the dynamical trajectory of that system. If the EEG is oscillatory, for example, the reconstructed trajectory of the study depicts a limit cycle. This type of analysis, however, is highly susceptible to noise, that may give the impression of low-dimensional dynamics and chaos in the EEG signal [11]. Many researchers have thus emphasized the importance of testing the validity of the reconstructed attractor. This is achieved by surrogate data testing methods where a surrogate EEG is designed to contain all the same spectral properties as the original data without including the nonlinear properties [12,13]. Nonlinear dynamical analysis is then applied to the surrogate and the original traces, and the difference in the analyzed results is used to determine whether nonlinear dynamics truly underlie the original data. If no difference between the original and surrogate data exists, then the original data contain no nonlinear features, and investigating the properties of the attractor, their dimensionality and their Lyapunov exponents would be unjustified. This provides a suitable control against the effects of noise and improves the validity of interpreting the results derived from the reconstructed attractor.

Normal and Pathological EEG

Current research suggests that normal, waking state EEG activity is characterized by weak nonlinear properties [14-17]. Studies of the alpha rhythm, observed during wakeful relaxation, have suggested the existence of two distinct types of activity detectable by nonlinear methods. Type I alpha activity is characterized by a point attractor whereas type II alpha reflects the dynamics of a limit cycle attractor [18]. The authors of the study suggest the type II alpha synchronizes large-scale activity in the brain and may switch to type I during cognitive tasks. Also, the nonlinear properties of the EEG were found to be modulated by the difficulty of a cognitive/attentional task. Specifically, the magnitude of the complexity of the EEG signal increased with the difficulty of the task [19,20]. Some authors have suggested that the parameters that control normal, resting-state EEG are likely close to the point where a bifurcation could occur [21]. This property permits the brain to flexibly shift its dynamics between oscillatory and non-oscillatory states in response to incoming stimuli. In nonlinear studies of sleep EEG, the presence of nonlinear dynamics was shown to characterize different sleep stages. One study suggests

that nonlinear features are strongest during stage II sleep when the sleeper is easily awakened [22]. Another study demonstrated that nonlinear analysis was more effective than linear methods at discriminating between stage I and II sleep [23]. Furthermore, levels of anaesthetic in the brain have been shown to correlate with the dimensional properties of the EEG signal where an increase in the depth of anaesthesia correlated with a decrease in the nonlinear correlation index [24]. This correlation was not observed with spectral methods. Overall, these studies suggest that the EEG signal in the normal brain is weakly nonlinear and is modulated by behaviour and stimuli.

In pathology, nonlinear methods have proven most useful in the study of epileptiform EEG epochs. There is convincing evidence that brain dynamics reduce complexity during seizures [25-27] but not during interictal periods, the time between seizures [28]. This change in the complexity of the signal has been observed 20 minutes prior to seizure onset [29] and has successfully predicted seizure episodes [30,31]. Interestingly, the level of complexity was increased by the administration of antiepileptic drugs [32], suggesting that the nonlinear properties of the EEG are strongly modulated by the underlying changes in brain activity in epilepsy. Some authors have suggested that the shift from ictal to interictal epochs reflects a bifurcation that arises from altered control parameters [33]. The altered balance of inhibitory and excitatory activity has been hypothesized to underlie the abnormal parameter range that causes the shift in the dynamics of the EEG [2]. While the epileptiform EEG is characterized by excessive synchrony, pathological changes in Alzheimer's disease produce drastic reductions in the synchrony and complexity of the EEG [34]. The EEG of Alzheimer's patients was shown to have less nonlinear structure than that of non-demented patients [35], and the dynamical response marked by flexible shifts between attractors, was shown to be impaired [36]. Synchronization of nonlinear features between different brain regions was also lower in Alzheimer's patients than in non-demented patients, and spectral, linear methods did not detect this diminished synchronization [37].

Conclusion

A review of recent studies suggests that the nonlinear properties of the EEG are strongly affected by disease states. Alteration or damage of neuronal networks appears to result in either hypersynchronous or random-like states that reflect pathology. High synchrony and low complexity characterize the epileptiform EEG, whereas low synchrony and low complexity characterize dementia. Nonlinear analytic methods provide a suitable tool for the detection of subtle phenomena that may otherwise be overlooked by spectral analytic methods. The further development of techniques and progression in the theoretical understanding of the relationship between various EEG attractors and underlying neural phenomena will provide interesting new insights into brain function during healthy and pathological states.

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JULS INTERVIEWS

Dr. Stephen Joseph Elledge

Dr. Stephen J. Elledge studied at the University of Illinois as an undergraduate and received his PhD in 1983 at the Massachusetts Institute of Technology (MIT) in Biology. In 1993 he became an Investigator with Howard Hughes Medical Institute and in 1995 was promoted to Professor. In 2003 he joined the Genetics Department at Harvard Medical School and the Division of Genetics at the Brigham and Women's Hospital. Currently, Dr. Elledge is the Gregor Mendel Professor of Genetics and Medicine at Harvard Medical School. In addition to a former Helen Hay Whitney Fellow, Dr. Elledge was an American Cancer Society Senior Fellow, and a Pew Scholar. In 2003 Dr. Elledge was also elected into the National Academy of Sciences and the American Academy of Arts and Sciences, to the American Academy of Microbiology in 2005, and the Institute of Medicine in 2006.

Interview conducted by Maya Deeb

"If you're satisfied, it's sort of like the end of science. It is the anxiousness and self criticism that keeps you trying to improve and take the next step."

MD: How did you become interested in studying cell cycle regulation?

SE: I became interested in cell cycle regulation after I clone ribonucleotide reductase. At that point it was known that the synthesis of nucleotides was cell-cycle regulated. And so, I though the enzyme I cloned is probably cell-cycle regulated…So I started reading about the cell cycle. That was around the same time that Paul Nurse and Leland Hartwell were working on cell-cycle regulation in *Saccharomyces cerevisiae.* There was a lot of interesting things to learn about. Paul Nurse had shown you could complement yeast CDC2 mutant, which is a key cell cycle regulator, with a human gene… so I thought that was great. But the tools at the time were sort of 'barbaric' – not very good. I improved on those methods and started cloning human genes involved in the cell cycle. Then I found another kinase, Cdk 2, and went on to find Cdk inhibitors and that is how it all started!

MD: What challenges did you face in this journey?

SE: Well… I think that there are lot of challenges. First of all, scientists are not trained to deal with people, they're trained to do science. There are a lot of challenges in running a lab and interating with students and educating them. Then there are a lot of challenges you face with yourself. For example, there are a lot of points in your scientific journey where you feel like 'is this the right thing for me? Should I do something else? Am I any good at this?'… And it's something just cyclical. Even the most serious and successful scientists have those doubts about themselves. It's this constant self doubt that drives. If you're satisfied, it's sort of like the end of science. If you're happy with where you are and complacent, you're just not drive. It is the anxiousness and self-criticism that keeps you trying to improve and take the next step.

MD: How important was mentorship in your career?

SE: It's important, I have to say it's important, especially when things aren't working. But, I was pretty independent after a couple of years of grad school. I had a pretty good graduate advisor. He was great, Graham Walker. But I was pretty independent. I think I was atypical in needing a lot of mentorship. I didn't need it, I was pretty much self-motivated and I knew what I was doing.

MD: Given the knowledge you established in the field of genetic instability, how do you see that applied in the future?

SE: Well, already there are some drugs that target some of the proteins that we discovered that they think are important to kill cancer cells. For example, Chk 1 inhibitors. The idea is that the pathway is partially debilitated by using this drug. You can push it over the edge by sort of, taking out another leg of the stool. At the point, the cell would die because it has too many things wrong with it, but the normal cells live… and that's only one way.

MD: Finally, what advice do you have for aspiring scientists, given that this is the audience of our journal?

SE: I just think that you should try to educate yourself broadly, and learn about all kinds of science. Because you never know what you're going to run into. You may run into a certain problem and the more familiarity you have with different branches of science from the one you're working in, the more likely you will be able to find a connection. The other thing is that, if you really set your mind to it, you can accomplish your goals. You don't have to come from a great background, or family or anything! If you have the desire and you're willing to work hard and really try to perfect yourself, you can get very far. You know, I'm not the smartest person in the world. I know a lot of smart people, much smarter than I am. But I am very determined and really driven to get what I want, and I believe other people can do that too!

"If you have the desire and you're willing to work hard and really try to perfect yourself, you can get very far."

MD: That is very inspiring! Thank you very much for taking the time to share with me some of your scientific journey! And congratulations again!

JULS INTERVIEWS

Dr. King Holmes

Dr. King Holmes is the William H. Foege Chair of Global Health at the University of Washington. He is also founder and director of the University of Washington Center for AIDS and STD, a World Health Organization Collaborating Center for AIDS and STD. Dr. Holmes completed his undergraduate degree at Harvard College in 1959, his medical degree at Cornell University Medical College in 1963 and received his Ph.D. in Microbiology from the University of Hawaii in 1967. Dr. Holmes is the principal investigator for the International Training & Education Center on Health (I-TECH), a collaborative program between the University of Washington and the University of California, one of the largest HIV/AIDS training programs in the world. He has participated in research on STDs for over 40 years in Africa, Latin America, Southeast Asia, and the Western Pacific. He has authored over 550 peer-reviewed publications and edited 30 books, monographs, and journal supplements.

Interview conducted by Nancy Liu

"The key is not having a 2 month rotation in another country. The key is learning about all of the factors that go into global health."

NL: So, you've had an undergrad at Harvard and an MD at Cornell and a PhD at the University of Hawaii, in all your years of academia, what are some of the most important things/lessons you've kept with you?

KH: At Cornell medical school, students had the opportunity to do a research project, and until then, I'd never really had any exposure to research. I had my lab courses, I was always being tested; it was interesting but not very much fun. Whereas the research project I had offered me a mentor, we would sit down and define a problem and try to discover something new. So it wasn't just a canned exercise that you were going through to try and figure out how to do a particular procedure. The one I got was to evaluate a potential new drug. It had been developed on the theory that pepsin not only aided in digestion, but might actually attack the stomach wall and cause ulcers. This was a putative ulcer drug that was supposedly going to inhibit pepsin. Now they had spent millions on this and I had a couple years. Now my studies wound up showing that the anti-pepsin activity of the drug was actually an artifact of the assay they had developed, there wasn't any activity from the drug.

During my internship at Vanderbilt, they had a usual rotation. Usually in internal medicine, you go through the wards and the clinics. This was my first rotation, and it was at the microbiology lab. And during your first rotation, you always put extra effort into it. So I wound up analyzing the anti-microbial susceptibilities of all the blood-stream isolates for several years by just sticking around after the lab closed. And I found that there are combinations of antibiotics which could cover all the major pathogens that weren't being used, and we were beginning to see some new resistance. So, it was just taking these old index cards, we didn't have computers in those days, and finding that there were these combinations of antibiotics that haven't been identified yet which we could use. I wrote that up as a 60 page paper with 600 references and gave it to my two mentors, who were very polite and thanked me. Then as I set off for the Navy, when they drafted me, my mentors kindly reduced my paper to 6 pages with 12 references and published it. So I learned a little bit from that.

When I got to the University of Hawaii, I bumped into someone who had been my TA at Harvard. I persuaded him to be my PhD thesis mentor, which I did on my own time while I was assigned to the Navy. It was by total good fortune I guess that I was able to

negotiate with the person responsible for assigning me. Instead of being assigned to a marine base in the desert of California, I was able to be assigned to preventative medicine. That was the only one with real world problems that I was interested in. And then, I was interested in infectious diseases because of my experience in the microbiology lab. There were real problems that required research to solve them. And I had gained some experience from medical school that gave me more confidence to do that.

"There were real problems that required research to solve them"

I guess one other thing I should mention is that I came from a very small town initially, White Bear Lake Minnesota, and wound up at Harvard and I didn't do so well in my preliminary exams. They had a program where they would teach the kids who didn't do so well how to speed read and the like. I would write stories and they would critique them for me. I really did learn how to write during that time.

So those were the main things: grounding yourself in the basics of reading and writing, and the opportunity to do some mentored research in med school, and negotiating for a reasonable opportunity in the navy. And that wound up being a valuable experience.

NL: Now, you're working on an international level, and you've been studying infectious diseases for the past 40 years. Have your initial thoughts about what drives you in your field changed/evolved? Anything astounding that you've learned over the years?

KH: Oh yeah, too many to mention! But I think some of the key things were that I was trained clinically, then I studied microbiology and got my PhD in that. Although I never formally studied epidemiology, I became the epidemiologist for the navy by assignment, so I had to study that on my own. So the ability to think across those domains made it particularly interesting and possible to identify a lot of people who could help. When I got back to the States to finish my residency in internal medicine, I wound up with a great mentor whose name is Marvin Turk. And he was a great clinician interested in infectious disease research. So that helped.

In terms of shifting paradigms, other than working across disciplines; when I was starting work at the University of Washington, I recruited people doing their post-docs, working in ob/gyn, neurology, internal medicine, pediatrics, pathology, epidemiology, biostatistics and other disciplines. And then they just started coming from other disciplines and I didn't have to recruit anymore. I think I had some big help from the NIH. I put in a grant proposal for a program project that had multiple areas of research in it, and it turned out that the director of the National Institute for Allergies and Infectious diseases thought that it was inappropriate for a second year faculty member to write a program project grant, so he came out to chair the site-visit review. He wound up liking the team, and he became a supporter at NIH for a long time. So I spent a lot of time reviewing grants at the NIH, even as a second year faculty member. Because there wasn't anyone else working in the STD field at the time, I had the opportunity to be a reviewer for all the grant proposals coming in for this emerging field. So I really got to see how to write a grant from being a reviewer and what were some of the interesting areas that were not being investigated well.

I think the big things that facilitated my work were having every department at the university involved in my research at some point. And I forgot to mention before other social science collaborators, like the anthropology department.

I think that I agreed to take a job as the Chairman of the Department of Medicine at our main teaching hospital for Harvard Medicine. I did that at the time just before HIV/AIDS just got started and really got going, and all the other STD's had gotten to such a low level. Although I had several grants, I thought it'd be more interesting to get into something more clinical. During that 5 year period, HIV/AIDS really became a huge problem. I decided to step-down as head of that department and pursued a HIV program. That grew pretty quickly, because we already formed this large faculty of people interested in STD's, it was pretty easy to shift the focus to HIV. And then it became evident, just from a couple of initial fellows going globally that there was much more of a problem in third world countries than in North America. So I took a sabbatical at the WHO, in the Global AIDS program in 1989-1990. I wanted to spend that time on the road and see what was going on from other UN agencies in 20 different countries. I evaluated what was really working, what wasn't working, what were the challenges, how bad was it, etc.

That led me to do much more work in global health. I then did a subsequent sabbatical at the London School for Hygiene and Tropical Medicine. I came back from there to propose to my university that we form a department of global health, and the strategic planning for this went on from basically 1990 to the end of 2002. And we put a proposal to the university to form the department. We then made a committee to interview all the stakeholders, potential students & faculty about how that department should be organized. Meanwhile I was doing much more of my work in Kenya, Peru, and Southern Asia.

After the initial experience with the Navy and the Philippines, then Kenya, and then Peru, finally the Gates Foundation gave us 30 million dollars to get started, and the State of Washington agreed to support us when we did an analysis of what was the cost of dealing with global health issues and how much money was being made from global enterprises.

Now Global Health is the second largest department at the University of Washington, after the department of medicine. We have a faculty from 15 of the 16 schools at the university as part of our department. And that's how I got into Global Health.

We have 500 students who are enrolled in our program now, including 5 Masters Programs and 2 Ph.D Programs (Global Health Basic Science, and Global Health Metrics and Implementation Science) and 5 Graduate Certificate programs. And we offer an undergraduate minor now.

It's been very much a student driven activity. Our student programs attempt to take people who have another area of expertise to bring into global health. I don't view global health as a discipline per se in the sense of epidemiology, medicine, or nursing. Once you've acquired skills in another field, you can then train with us and be able to apply your skills in global health.

We followed 400 of our alumni, and essentially all of them are employed. We didn't know when we started if there was going to be a market for people in global health. Although we've only been going for 7 years, our graduates are finding work. It tends to be in academia or business in terms of basic sciences, and in non-laboratory settings, also academia, as well as NGO's and Ministries of Health. About half of our students come from other countries, and about half from the US. So we have a good mix. If you go to our website, which I encourage, look up University of Washington Department of Global Health. The website will show an interactive map and you can click on any country. We have about 742 programs in 114 countries. You can click and see who's working where and what they're doing. Gives you a sense of what's going on in the department.

NL: That's really impressive! What's your prognosis then on the future of Global Health?

KH: Well I think it's a good field to get into. When I started, William Foege was the director of the CDC. He then stepped down from that to lead to the smallpox eradication efforts. He was one of the key players, he wouldn't claim responsibility, but is credited with eradication of smallpox from part of West Africa and India. He's a real visionary and has chairs in my external advisory board. He said when we built the Foege building at the University of Washington, that the biggest problem you're going to have working in this building is learning how to pronounce the name. What he told me when I started was, "This is field is going to last for at least 25 years." And that was 6 years ago. So I figure we have a few years left.

"...every course whether it's community health or cardiovascular disease should talk about what's unique in different countries."

Of course he didn't know. And I would say, if anything, it's rolling. Of course this depends on the world's economy, politics, and the extent to which we continue to be supportive of global health programs. I think what one of the most interesting things we're doing here is to attempt to globalize our curriculum. I think that the future will be diverse. I think we'll be evolving more towards is taking more focused programs and evolving them into programs that learn to develop health systems that enable our partner countries to build systems that take into account prevention, healthcare, policy, etc. that crosses these small territorial borders into broad issues that include health outside the developed world. To be able to think at the level of overarching principles, policies, educational systems, integration of systems, interprofessional education, and interdisciplinarity. These factors are key.

NL: Lastly, do you have any advice for our students hoping to go abroad or enter into the field of global health? What kind of attitude should they come in with?

KH: Aim for a place that offers a program that is broad that can use the skills you've acquired. The key is not having a 2 month rotation in another country. The key is learning about all of the factors that go into global health. An example in terms of globalizing medical school curriculum: every course whether it's community health or cardiovascular disease should talk about what's unique in different countries. Such as, emergency response, general knowledge of symptoms–some places people aren't aware chest pain could signify a heart attack—access to a primary health clinic and the technology available. It's important to find out what is being done and what is actually feasible. Question what is the role of new diagnostic tests, what policies should be there to apply point-of-care diagnostics as they evolve? To really assess the needs and the capabilities of each area you could be interested in. To have in mind the greater view of what a system could look like to do a better job of preventing or treating a certain disease.

I suggest students look for a program that isn't narrow, that has lots of opportunities, that really focuses on student education, and gives a wide scope of training related to global health. And always, overseas study is an excellent opportunity if it's available to you.

Do your research about the universities and programs you apply to. Identify who has meaningful projects going on in whichever country you'd like to apply to. Look for long term interests that might match yours. Network and talk to lots of people!

NL: Well thank you very much for speaking with me about your experiences! And congratulations!

Sir Gregory Winter

Sir Winter completed his studies in Chemistry and Biochemistry at Trinity College, Cambridge University. His pioneering work in humanized monoclonal antibodies has been implemented in cancer as well as immunological disease treatments. Sir Winter was the founder of three biotechnology companies that has developed some of the most successful antibody drugs and molecular therapies in use today. He is a Fellow of the Royal Society, the Master of Trinity College, recipient of the Royal Medal, King Faisal International Prize, Cancer Research Institute William B. Coley Award, and the 2013 Gairdner International Award.

Interview conducted by Lucy Chau

"In a way, I didn't set up these companies to set up a company, they were how I moved forward with my experiments."

LC: So you did your graduate work in chemistry and biochemistry at Cambridge. How did you first become interested in Antibody Engineering?

GW: I've been doing enzyme engineering at Cambridge and I was interested in figuring out novel enzyme bind sites and interaction sites. I naturally began to look into structure of the antibody.

LC: When did you realize that you experiments with monoclonal antibodies could become marketable products?

GW: We created a sort of a model. I suppose the question was when did I "realize" right? Obviously, I wouldn't start the experiment if I didn't think it would have potential. I mean, it takes 2 years to make those antibodies. I guess the key thing was when we started making the humanized antibodies. This lead to very promising results in the in vitro model and also great results in the in vivo model.

LC: Did you expect your humanized monoclonal antibodies would become such a success, both commercially (with billions of dollars in annual sales) as well as medically?

GW: Umm, no. I thought the antibody would be very useful, especially for cancer. But I certainly did not think it would be applicable in so many immune disorders. I thought it would mainly work best for killing cells like for diseases in which cells are overtly proliferating. So that's where I thought it might go at the time. In fact, I didn't really think about its use as a disease treatment until much later.

LC: You founded three companies: Cambridge Antibody Technologies, Domantis, and most recently Bicycle Therapeutics. How did you balance your academic pursuits with your entrepreneurial ambition?

GW: Well one thing was to be consistent in your interests. I was motivated to start a company using this new antibody technology. And also, I was personally devoted to using this and saw it as an opportunity to gain more firepower.

LC: What do you mean by firepower?

GW: Well, I mean more post-doc positions. Ability to compete with some of the giants in the industry. So that's what I mean by firepower. When you're doing stuff like we are, that has

therapeutic potential, you have to validate what you find in the laboratory. You need to move forward from your experiments because otherwise, this is just a curiosity that you're pursuing. In the end, you need to find a way to do it. One way would be to develop a clinical trial, surgical trial, and ask for funding. Another will be to propose this to an existing company, but no one's willing to pay for it. So in the end, you need to set up a company. In a way, I didn't set up these companies to set up a company, they were how I moved forward with my experiments.

"If you know something is wrong, be flexible. Change it; change with your ideas."

LC: Do you think the future innovations in the biotech industry will come from small start-ups like yours?

GW: Well, it has been for a while. But on the other hand, the large pharma companies have adapted. They're not stupid. The issue is, if you've got a disruptive technology. The kind of market leaders—the big companies might not see it. Think like Microsoft and IBM, established giants. They didn't see it coming from the likes of Apple. For example, a similar thing to think about would be microchips that are in most of your devices today are from a Cambridge company called ARM. At the beginning of this technology, none of the establishment companies were interested in this. They were focused on their existing technologies and used to their own way of thinking. And of course they have to focus on their own projects because they have such a huge market share. So this is like the antibody technology today with the larger companies. They ask me: "What do you think the market is like? What do you think the sales are like?" Well to be honest, I don't know! When you create a new market, it's the same as before the ipad came in. You have novelty and you create the market for the product. It was not obvious what it could be used for. Before, pharmaceutical companies deal with existing problems using small molecules. They didn't feel the need to deal with antibodies and they don't understand the issues involved. I mean, they're so complicated, you have to ferment them, they're hard to make, they don't understand its biology. Nobody thought it was possible to work with them for a hundred years. It took small groups to make the concerted effort to make it work and it did!

LC: And finally, what advice do you have for the aspiring scientists, immunologists, or entrepreneurs in moving carrying forward their translational research?

GW: Well for me, it would be to make sure you are a really good basic researcher. You have to make sure you are an absolutely proficient scientists. I'm not talking about a scientific manager, who doesn't need to have absolutely strong science skills. I'm talking about someone starting from a scientific background moving into a more commercial field. You need to make sure you really are the knowledge expert. First of all, only when you have solid experience will you know what sort of experiments to do. Second of all, nobody will believe what you propose unless you have such an established knowledge base. So that was the first requirement. Secondly, you need to be utterly self-critical. It's very easy to mislead yourself. Things now that appear very clear are not always clear at that time. You have lots of things going on, lots of distractions. Very often, your first ideas need to be improved. Your original idea of what you base your company on is not what it always ends up. You will get some revelation and everything will change. You have to be flexible. If you know something is wrong, be flexible. Change it; change with your ideas.

LC: Do you think mentorship and collaboration has a role in developing these new ideas?

GW: Well yes! I was very lucky to have great mentorship on the scientific side. I didn't have as much support on the commercial side, apart from some Australian collaborators. So it's very important to have support, especially enthusiasm for your ventures.

LC: That is all the questions I have for you right now. Thank you so much for your time!

GW: No worries, it was a pleasure.

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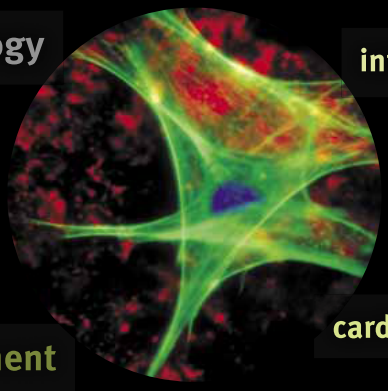

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