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Does dysregulation of key epigenetic and biochemical pathways occur in postulated vasoactive neuropeptide autoimmune disorders?

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Summary Autoimmune dysfunction of certain vasoactive neuropeptides (VNs) has been postulated as a contributing cause of sudden infant death syndrome (SIDS), chronic fatigue syndrome (CFS), Gulf War syndrome (GWS) and other fatigue-related disorders. This family of VNs includes pituitary adenylate cyclase activating polypeptide (PACAP), vasoactive intestinal peptide (VIP) and calcitonin gene related peptide (CGRP). The postulated mechanism is compromise of adenylate cyclase activation, a vital and unique step in cyclic AMP production from ATP, through autoimmune dysfunction of VNs, their receptors or their genes possibly involving cytosine-phosphate-guanine (CpG) fragments.

CpG fragments are immunomodulatory dinucleotides serving as 'friend or foe' recognition systems to differentiate bacterial and viral (hypomethylated CpG) from mammalian (methylated CpG) DNA. However hypomethylation disorders affecting these fragments in mammals may convert them to dysfunctional states by promoting autoimmune inflammatory reactions. Epigenetic mechanisms acting on gene promoter regions may contribute to the development of VN autoimmune fatigue-related disorders through CpG fragments located in vital segments of VN/receptor genes by causing signalling defects with profound implications for VN function. Neurotransmitter dysfunction particularly glutamatergic transmission could also result with disruption of neuronal cellular biochemical functions such as ammonia regulation. Endosomal acidity and mitochondrial membrane potential modifiers such as chloroquine, together with immunoregulatory therapies, may have therapeutic implications in protecting against these apparent autoimmune disorders.

This paper examines specific epigenetic and biochemical mechanisms possibly mediated by VN or receptor genes resulting in postulated VN autoimmune fatigue-related disorders. These mechanisms may have implications for treatment and prevention options for VN autoimmune disorders. VN autoimmune processes have implications for military medicine where radiological, chemical and biological agents may play an important role in pathogenesis.

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Background

Vasoactive neuropeptides (VNs) including pituitary adenylate cyclase activating polypeptide (PACAP),

vasoactive intestinal peptide (VIP) and calcitonin gene related peptide (CGRP) comprise a class of bioactive neuropeptides exerting multiple effects in hormonal, neurotransmitter, neuroregulatory, neurotrophic, immunomodulatory and metabolic systems. They are related to the secretin/glucagon family and they and their receptors have substan-

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tial sequence homology. Autoimmune dysfunction of these substances or their receptors is postulated to contribute to VN fatigue-related disorders including sudden infant death syndrome (SIDS), chronic fatigue syndrome (CFS) and Gulf War syndrome (GWS) [1].

This family of VNs are adenylate cyclase (AC) activating peptides. ACs comprise a family of around 10 isoforms in mammals, operating via G protein-coupled receptors (GPCRs). A postulated autoimmune mechanism is through compromise of AC activation, a vital and unique step in cyclic adenosine monophosphate (cAMP) production from adenosine triphosphate (ATP). Signalling systems including protein kinase A, phospholipase C and calcium may also be involved [2]. Only three receptor types have been identified for PACAP and VIP, namely PAC1, VPAC1 and VPAC2, although functional and non-functional variants have been identified [3]. CGRP is mediated via different receptors.

This paper describes some epigenetic and biochemical mechanisms likely to contribute to the development of postulated vasoactive neuropeptide autoimmune fatigue-related disorders. Epigenetic dysfunction of cytosine–phosphate–guanine (CpG) fragments located in vital segments of VN receptors or their genes may lead to signalling defects with profound implications for VN function. This would result in defective activation of AC with subsequent defective cAMP activation and signalling disruption for this important secondary intracellular transmitter. Impaired control of inflammatory responses would result via NFkappaB and other nuclear transcription regulators of inflammation. Neurotransmitter dysfunction would also result (e.g., glutamate) with disruption of neuronal cellular function. Endosomal acidity modifiers such as chloroquine may have therapeutic implications in protecting against these apparent autoimmune disorders.

Epigenetic mechanisms in vasoactive neuropeptide gene control

DNA CpG fragments are immunomodulatory fragments serving as 'friend or foe' recognition systems to differentiate bacterial and viral (hypomethylated CpG) from mammalian (methylated CpG) DNA. They exhibit significant immune responses [4]. Hypomethylation disorders affecting these fragments in mammals may give rise to autoimmune dysfunctional states by promoting inflammatory responses. CpG fragments exist in vital

segments such as promoter regions of VN and VN receptor genes [5,6]. Constitutive methylation-hypomethylation activities may also influence or regulate genetic expression of VNs and their receptors with implications for gene expression. Mistakes of recognition may result from bacterial and viral infection, or other hypomethylation causes such as chemical, radiological or spontaneous activity adversely influencing epigenetic regulation of these VN genes.

Initiation of VN autoimmune responses via CpG fragments therefore has been postulated [7] and may have implications for prevention and treatment of VN disorders [8]. Moreover, occurrences of bacterial and viral infections exhibiting molecular mimicry with certain CpG fragments may account for the epidemicity sometimes observed in CFS, hence the term epidemic myalgic encephalomyelitis (EME) [9]. CpG-related epigenetic mechanisms thus have the potential to result in VN autoimmune fatigue-related disorders. CpG-rich fragments are also high affinity binding sites for several nuclear factors [10] indicating mechanisms involving inflammatory responses.

CpG fragments regulate VN and receptor genes hence aberrations of their function are likely to occur from CpG malfunction. Broad et al. [11] note that de-methylation acts as a mechanism to regulate gene expression in calcitonin- α CGRP. Such a mechanism could prove to be critical in causing VN autoimmune disorders. Aino et al. [12] have identified CpG-rich fragments in the proximal promoter region of the PAC1 receptor gene indicating a plausible site for PAC1 receptor expression dysfunction. VN autoimmune dysregulation would have important implications for VN control of inflammatory processes as these substances have powerful roles in inflammatory protection [13,14]. These studies indicate compelling forensic opportunities for VN dysfunction through CpG autoimmune mechanisms.

CpG fragments and autoimmunity

Autoimmune dysfunction of CpG fragments may occur where hypomethylation occurs promoting a 'foreign recognition' signal. Such hypomethylation may result from infectious mechanisms where self-DNA is mistaken for bacterial fragments (e.g., via molecular mimicry), from chemical and radiological causes, and possibly spontaneously. Klinman et al. [15] note the propensity of CpG fragments to initiate stimulatory autoimmune effects, as well as mediating suppressive mechanisms.

These effects are mediated via toll-like receptors (e.g., TLR9) which also serve as foreign bacterial recognition systems.

He et al. [16] found that CpG DNA released during infections may exacerbate autoimmunity by stimulating autoreactive B cells to switch from an IgM to a more pathogenic IgG isotype. Hence as well as regulating expression of TLRs, CpG fragments influence B and T cell clonal expansion, thus setting in place long-term immunogenic responses including class switching. He et al. also found that B cell activation by CpG DNA occurs via a TLR9 mediated NFKappaB dependent innate pathway cooperating with IL10 and STAT proteins and IFN responsive factors. He et al. note this pathway is blocked by chloroquine which is known to attenuate manifestation of IgG mediated autoimmune disorders.

Hong et al. [17] found that chloroquine protects against CpG oligodinucleotide (ODN) and lipopolysaccharide (LPS) challenge by decreasing the promotion of cytokine release. Park et al. [18] however note that inflammatory regulation may be increased or decreased depending on the cellular context. Yi et al. [19] note that chloroquine abolishes CpG DNA mediated protection against spontaneous apoptosis of splenic B cells and that CpG acts by preventing mitochondrial membrane disruption, suggesting chloroquine may provide a possible mechanism to overcome long term promotion of potentially pathogenic B cells.

In addition to determining causal mechanisms of pathological autoimmune responses to CpG and vasoactive neuropeptides, these observations have important treatment and prevention implications through drug, epigenetic and anti-idiotypic antibody therapies. Immunogenicity against VNs, CpGs and heat shock proteins (hsp) [20,21] may be converted from short-term and relatively benign IgM responses to more pathogenic and long-term IgG immunological responses. Perverse immunological memory created against the VNs or their receptors would result in an extraordinary array of disorders including central and peripheral fatigue, disturbances of cerebation, sleep and behaviour as well as brainstem and autonomic regulation, chemosensitivity, neurotransmitter and gaseous neurotoxic and reactive oxygen species sequelae. Hence elucidated mechanisms for CpG, hsp and TLR activation may have applications in treatment and prevention of VN autoimmune disorders. Suppressive ODN and drugs such as chloroquine may have a role in these potential therapies as both influence TLR signalling. Clearly complex biochemical pathways are likely to be involved in the pathophysiology of these postulated conditions and are considered further below.

Dysregulation of multiple complex biochemical pathways

Multiple complex biochemical pathways are mediated by AC activating VNs. Some key pathways are considered below but are not exhaustive. Effector pathways for apoptosis are considered along with glutamate metabolism as a model for dysregulation of vital neurotransmitter and biochemical mechanisms postulated in VN autoimmune disorders. These pathways exemplify mechanisms operating in conditions of cellular and somatic stress within and beyond the central nervous system (CNS).

PACAP may be a potent mediator of the stress response to certain stimuli [22]. Delgado [23] reports inhibition of the MEKK1/MEK4/JNK pathway, leading to a reduction in phosphorylated c-Jun and stimulation of JunB, mediated through the VPAC1 receptor via the cAMP/PKA pathway. VIP/PACAP interference with the stress-induced SAPK/JNK pathway in activated microglia may thus represent a significant element in the regulation of inflammatory responses in the CNS by endogenous neuropeptides.

PACAP and VIP are potent neurotrophic substances and play a vital role in neuronal survival. PACAP inhibits apoptosis in many tissues including cerebellar granule cells by inhibition of caspase-3, and mitochondrial pathways play a pivotal role in these anti-apoptotic effects [24]. PACAP acts by strongly inhibiting C2-ceramide-induced activation of caspase-3 [25]. Ceramide-induced apoptosis is inhibited in PC12 cells by PACAP by affecting signalling downstream of JNK activation [26]. Moreover, PACAP has been shown to stimulate MAPK in both PKA- and PKC-independent manner in astrocytes [27]. Ceramide (C2) mitochondrial potential inhibitory effects mediated via caspase systems together with cytochrome c release from mitochondria are countered via PACAP in apoptosis [28]. VIP also inhibits translocation of cytochrome c from mitochondria in hippocampal cells in protecting against apoptotic cell death [29]. Hence, the implications for VN failure are serious as PACAP/VIP play a critical role in mitochondrial pathways in protecting against apoptosis.

Complex biochemical pathways intersect immune and neurotransmitter functions and are modulated by VNs and glutamate serves as a useful model. For example, exposure to ammonia during prenatal and lactation periods results in long-lasting impairment of NMDA receptor function which may be associated with altered aspartate aminotransferase activity [30] and hence altered

glutamate function which may be relevant in SIDS. Lee et al. [31] note significant differences for alanine/aspartate transaminase (AAT) and gamma glutamyl transaminase (GGT) in blood tests in Gulf War veterans. *N*-methyl-D-aspartate (NMDA) is known to have a trophic effect on cerebellar granule cells [32] and is known to enhance activity of AAT considerably [33]. NMDA may in turn be modulated by cAMP which is induced by PACAP. [34]. PACAP is able to enhance NMDA receptor function and also enable RACK1 expression of brain-derived neurotrophic factor (BDNF) [35]. Hence loss or compromise of function of PACAP would be expected to have predictably significant effects on NMDA and neuronal function. These findings have significant implications for glutamate neurotransmission.

Intra- and extra-cellular calcium regulation appears to be vital in VN function. Dziema and Obrietan [36] note that PACAP potentiates L type Ca(2+) activity. Suprachiasmatic nucleus neurons become sensitive to glutamate only after PACAP administration, suggesting that PACAP sets the lower concentration threshold required for glutamate to initiate a robust rise in postsynaptic cytosolic Ca(2+). The modulatory actions of PACAP are related to the p42/44 mitogen activated protein kinase (MAPK) signal transduction cascade. Aoyagi and Takahashi [37] note that PACAP enhances Ca(2+) dependent glutamate neurotransmitter release in PC12 cells by modulating steps subsequent to Ca(2+) entry. Chen et al. [38] note that ATP increases Ca(2+) by mobilising internally stored Ca(2+) followed by an influx of Ca(2+). Defer et al. [39] note that AC is tissue specific particularly in relation to Ca(2+)/calmodulin functions and that signals received by GPCRs can be differentially integrated. Hence, calcium regulated by VNs plays a key role in cellular and neurotransmission functions.

Receptor function is also vital in coordinated and integrated VN activity. Nowak and Zawiska [40] note that the plethora of GPCRs and the functional differentiation of G-protein subunits and many AC isoforms permits a very complex signalling system with a wide variety of integrative characteristics. Chabardes et al. [41] note AC types 5 and 6 constitute a sub-family having the property of being inhibited by submicromolar Ca2+ concentrations in addition to Galpha(i)-mediated processes. This ensures wide changes in cAMP synthesis. Mons et al. [42] note AC types 1 and 8 stimulate Ca2+/calmodulin in the hippocampus and this suggests a role for hippocampus-related memory function. Chern [43] note that AC isoenzymes are tightly controlled by various signals and one of their most important impacts is on the complexity and fine-tuning of

cellular signalling especially in the CNS where multiple signals constantly occur.

A complex relationship exists between VNs and their immunological, biochemical, neurotrophic and neurotransmitter modulating functions. Shaked [44] note that T cells reactive to CNS-specific self-antigens protect neurons against glutamate toxicity. Antigen-specific autoimmune T cells increase the ability of microglia-enriched cultures to remove glutamate. This up-regulation of glutamate uptake induced by IFN- γ activation is not accompanied by the acute inflammatory response seen in LPS-activated cultures. Hence, T cells or their cytokines can cause microglia to adopt a phenotype that facilitates rather than impairs glutamate clearance to contribute to restoration of homeostasis. Kuratsune et al. [45] note that CFS patients have reduced acetylcarnitine uptake in the brain and they have lower serum levels. Acetylcarnitine is mainly used in the biosyntheses of glutamate. This might imply suppression of the glutamate bio-syntheses system possibly because of glutamate overload. Yao et al. [46] note that astroglial cells group II and III metabotropic glutamate receptors (mGluRs) exert neuroprotective effects through enhancement of glutamate uptake. Molz et al. [47] note that glucose-deprived rat slices induce a significant decrease in ATP which is unchanged by addition of glutamate or GMP. This may provide further evidence for ATP accumulation and toxicity in VN dysfunction as ATP might then be accumulated rather than converted to cAMP via adenylate cyclase.

VNs mediate a large number of neurotransmitters centrally and peripherally, including glutamate [48]. Glutamate is an important excitatory and modulatory neurotransmitter and excitotoxicity may occur due to VN autoimmune dysfunction. Glutamate is reversibly synthesised from glutamine via glutamine synthetase (GS) and metabolises to α -ketoglutarate and ammonia, an important process for ammonia metabolism and disposal. In turn GS is activated by α -ketoglutarate to prevent accumulation of ammonia, a vital step in preventing ammonia toxicity. Hence, dysfunction of glutamate regulation will have important implications for ammonia metabolism. Interestingly, glutamic acid in the second intracellular loop of the PACAP receptor may be a key residue to constrain the receptor in the inactive conformation with respect to its coupling to G(s) proteins [49].

Rangon et al. [50] note that VIP has a protective effect for glutamate via the VPAC2 receptor. White matter excitotoxic lesions were induced by the glutamate agonist ibotenate in neonatal mice and are similar to periventricular leukomalacia. Shintani et al. [51] noted PACAP mRNA levels were

increased up to 3.5 times 8 h after glutamate exposure in rat neuronal cultures indicating a neuroprotective role of PACAP. Moreover, Dong et al. [52] noted the role of intracellular calcium regulation by PACAP as a mechanism to control glutamate toxicity in hippocampal neurons. Glutamate transporters have a vital role in 'clearing' glutamate from the extracellular environment and absorbing it via astrocytes to protect neurons from toxicity [53] and these transporters are potently activated by PACAP [54,55]. Both glutamate and PACAP also control clock genes in regulating diurnal rhythm and sleep-wake cycles and code light and dark information [56]. Hence, PACAP and VIP exert significant control over glutamate in key neuronal functions which would be severely compromised if these VNs undergo autoimmune dysregulation. These studies illustrate the critical roles PACAP/VIP vasoactive neuropeptides have in mediating cAMP formation via a complex array of ACs. Failure to carry out these functions has potentially catastrophic outcomes, particularly in neuronal systems such as hippocampus, by unopposed caspase induced apoptotic cell death via mitochondrial-sensitive pathways.

PACAP plays a critical role in protecting tissues from hypoxia as well as regulating gaseous neurotransmitters. Suk et al. [57] note that PACAP protects microglia from ischaemic effects of hypoxia. Indeed Rabl et al. [58] note that turtles have much greater levels than mammals to protect them from diving induced hypoxia. Cummings et al. [59] have found that PACAP deficient mice die from a SIDS-like syndrome as neonates and Wilderman [60] notes the role of PACAP and cAMP in opioid mediation in hypoxia induced pial artery dilatation, suggesting nociception modulating influences. The gaseous neurotransmitters nitric oxide (NO) and carbon monoxide (CO) play vital roles in cellular metabolism and are tightly regulated by VNs to preserve homeostasis. They are postulated to be associated with SIDS because of their known association with cigarette smoking. Martinez [61] notes the role of the PAC1 receptor in nitric oxide signalling and septic shock. Hence, VNs have complex and crucial regulatory functions of vital gases and gaseous neurotransmitters.

Other pathways and functions

PACAP and VIP are related to the secretin/glucagon family of hormones. They have a vital role in fine-tuning insulin signals and hence may operate as insulin enablers or suppressors. PACAP and VIP also

exert significant influence over almost all major neurotransmitters. These include cholinergic, adrenergic, noradrenergic, histaminergic, serotonergic as well as glutamatergic systems. A multi-system constellation of consequences results with profound disturbance of homeostasis manifesting as fatigue and other significant CNS and peripheral effects. However, a full discussion of these effects in postulated VN autoimmune disorders is beyond the scope of this paper.

Conclusion and future directions

Epigenetic mechanisms related to autoimmune-sensitive segments of VN and VN receptor genes may play a significant role in postulated VN autoimmune disorders. These mechanisms reflect the propensity for CpG-rich fragments to undergo hypomethylation as a result of a variety of immunological, chemotoxic, infectious or radiological assaults and assume bacteria-like qualities. Hence, autoimmune reactivity is a possible outcome resulting in mistaken CpG recognition of 'self' sequences as 'foreign'. Neurotransmitter disturbances are a likely consequence of this autoimmune activity and glutamate serves as a useful model for neurotransmitter dysfunction.

Elucidation of postulated VN autoimmune fatigue-related conditions will have important implications for treatment and prevention and will include both physical and psychological therapies. Individuals may be severely incapacitated by these conditions and significant physical and behavioural impairments result in major disruption to their lives. Vital areas such as hippocampus, limbic system, deep nuclei, brain stem and autonomic systems are affected as well as smooth and skeletal muscle functions because of the known relatively high VN and/or receptor concentrations in these tissues and their role in a wide array of neurological, immunological and metabolic functions.

A spectrum of interventions including genomic, immunological and biochemical/drug therapies may prove to be possible in VN autoimmune fatigue-related disorders. The concept of prevention of SIDS if shown to be a VN autoimmune disorder may also evolve in this way. Public health implications may exist if 'epidemics' or simply seasonal circulating organisms have particular molecular mimicry with VNs or their receptors. This is reflected in the term 'epidemic or benign myalgic encephalomyelitis'. Short term relatively benign IgM may shift to a more pathogenic IgG phenotype as autoimmune responses to VNs/receptors and re-

sult in longer term profound impairment and disability. VN and receptor reactivation may prove to become successful interventions. These VN autoimmune processes have implications for military medicine where radiological, chemical and biological agents may play an important role in pathogenesis.

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