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OPINION ARTICLE

Could Small Neurotoxins-Peptides be Expressed during SARS-CoV-2 Infection?

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Abstract: SARS-CoV-2 pathogenesis has been recently extended to human central nervous system (CNS), in addition to nasopharyngeal truck, eye, lung and gut. The recent literature highlights that some SARS-CoV-2 spike glycoprotein regions homologous to neurotoxin-like peptides might bind to human nicotinic Acetyl-Choline Receptors (nAChRs). Spike-nAChR interaction can probably cause dysregulation of CNS and cholinergic anti-inflammatory pathways and uncontrolled immune-response, both associated to a severe COVID-19 pathophysiology. Herein, we hypothesize that inside the Open Reading Frame (ORF) region of spike glycoprotein, the RNA polymerase can translate small neurotoxic peptides by means of a "jumping mechanism" already demonstrated in other coronaviruses. These small peptides can bind the snAChRs instead of Spike glycoproteins. A striking homology occurred between these small peptides observed by sequence retrieval and proteins alignment. Acting as nAChRs antagonists, these small peptides (conotoxins) could be the explanation for the extrapulmonary clinical manifestations (neurological, hemorrhagic and thrombotic expressions, the prolonged apnea, the cardiocirculatory collapse, the heart arrhythmias, the ventricular tachycardia, the body temperature alteration, the electrolyte K+ imbalance and finally the significant reduction of butyryl cholinesterase (BuChE) plasma levels, as observed in COVID-19 patients. Several factors might induce the expression of these small peptides, including microbiota. The main hypothesis regarding the presence of these small peptides opens a new scenario on the etiology of COVID-19 clinical symptoms observed so far, including the neurological manifestations.

Keywords: SARS-CoV-2 genome, infection, neurotoxins, acetylcholine, sequence alignment, microbiome.

1. INTRODUCTION

The COVID-19 pandemic, a global emergency due to SARS-CoV-2 infection, has resulted in 187,827,660 confirmed cases of infection and 4,055,497 deaths from beginning to date (World Health Organization - WHO) [1]. The presence of previous chronic diseases represents a significant risk factor and influences the prognosis as making patients more vulnerable to COVID-19 disease, from progression towards worsening outcomes [2]. In addition, other lifestyle-related risk factors such as physical inactivity, obesity, excessive alcohol intake and smoking, have also been proposed [3, 4].

Particular attention has been devoted to smoking, as evidence indicate that smokers with COVID-19 are more likely to have serious illness and adverse outcomes once hospitalized [5]. However, the number of smokers requiring hospitalization is far lower than expected, according to population smoking rates [5, 6-9]. Since the beginning of the pandemic, several studies have shown a negative association between smoking prevalence and COVID-19 hospitalized patients, leading some researchers to hypothesize a possible therapeutic role of nicotine in the course of SARS-CoV-2 infection [7, 10, 11].

Based on these observations, some research groups have suggested an interaction between the human nicotinic acetylcholine receptor (nAChRs) and SARS-CoV-2virus, in line with the finding that viral Spike glycoprotein has specific motifs related to known nAChR antagonists [5, 7, 8, 11].

SARS-CoV-2 virus, initially isolated from ocular secretions of COVID-19 affected patients [12], is part of β-coronaviruses' group and contains a single-stranded positive RNA (~ 29.9kB) with 14 Open Reading Frames (ORFs) encoding for 27 different proteins [13, 14]. Spike glycoprotein is expressed on the surface of virus envelope, representing a fun-

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damental key point to mediate the entry of SARS-CoV-2 into host cells by recognition of human ACE2 (hACE2) receptor through a region defined as Receptor-Binding Domain (RBD) [15, 16].

Using a molecular dynamics computer simulation based on data from a previous study by Changeux and coworkers, Oliveira and coworkers identified i. a region on Spike protein ranging from amino acids 674 to 685 with high homology to several neurotoxins as α-bungarotoxin, a nAChR antagonist directly competing with acetylcholine identified in the snake Bungarus multicinctus, and ii. a high affinity region for the human α4β2 and α7-nAChR subtypes [8, 11]. The same research team also hypothesized the interaction of spike protein with α7-nAChR [8, 11].

By sequence alignments, Farsalinos and coworkers identified homology between the Spike Glycoprotein area between the aa 375-390 and the neurotoxin homolog NL1. This *in-silico* study suggested a strong interaction of the amino acid motif with the nAChR α9 subunit [17]. The aa 375-390 peptide fragment is included in the RBD spanning aa 319-541, a domain that enables the spike protein to recognize ACE2 on the host cells [17]. Further advanced *in-silico* studies by the same group highlighted that the sequence of the aa 375-390 corresponded with the previously described cryptic epitope for human anti-SARS-CoV antibody CR3022, and through a particular and unusual folding of Spike glycoprotein, it might also bind to α7-nAChR [6].

The involvement of the nicotinic cholinergic system might explain some aspects of COVID-19 pathogenesis not well understood to date. Numerous evidence suggests a neurotropic action of SARS-CoV-2, a point also confirmed by a recent study in which it was demonstrated how the virus could penetrate through the olfactory mucosa and follow the neuro-anatomical structures reaching the primary respiratory and cardiovascular control centers of the medulla oblongata [18]. Retrospective studies have demonstrated neurological manifestations in more than 30% of COVID-19 patients with Central Nervous System (CNS) symptoms such as dizziness, headache, impaired consciousness, acute cerebrovascular disease, ataxia, and seizure, in addition to the loss of smell and taste that are specific to this disease [19].

Activation of α7-nAChRs, particularly expressed by B cells, T cells, and macrophages, reduces the production of proinflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) through a "cholinergic anti-inflammatory pathway" triggered by nicotine or nicotinic agonists [9, 20]. This nAChR antagonistic action of virus would therefore explain the "cytokine storm" and the hyperinflammatory syndrome observed in several COVID-19 patients [9, 11]. Numerous authors also hypothesized a prominent role of macrophages highlighting that the antagonistic action on α7-nAChRs by SARS-CoV-2 determines a dysregulation of the polarization mechanism and a permanence of M1 type macrophages, with a consequent increase in the concentration of inflammatory cytokines [21].

Finally, it is interesting to note that α7-nAChRs are also present on the surface of platelets and that, as previously demonstrated, their inhibition can lead to an increase in their activity and consequent clot formation, thus explaining coagulopathy and thrombus formation in COVID-19 patients [9, 11].

Recently, the direct effect of inhibition of COVID-19 on critical survival genes has also been hypothesized, such as Sirt 1, that are linked to expression levels of α7-nAChRs [22, 23], resulting in effects that can range from inflammatory processes to programmed cell death [24].

2. SEQUENCE ALIGNMENT ANALYSIS

We mainly focused on the previously mentioned alignment studies that demonstrated a sequence homology between some regions of the virus Spike glycoprotein and nAChR antagonists such as α-bungarotoxin [8, 11] and Neurotoxin homolog NL1 [5, 6].

To verify the similarity between SARS-CoV-2 Spike glycoprotein sequence [6] and different neurotoxins with nAChR antagonistic action, we used EMBOSS Needle, a bioinformatic sequence analysis application provided by European Molecular Biology Laboratory - European Bioinformatics Institute (EMBL-EBI) (https://www.ebi.ac.uk/Tools/psa/emboss_needle/) [25, 26].

EMBOSS, acronym for European Molecular Biology Open Software Suite, is a free open-source software analysis package and contains a wide array of general-purpose bioinformatics programs for the needs of the molecular biology user community. Among these, EMBOSS Needle tool reads and compares two input sequences along with their entire length and writes their optimal global sequence alignment to file. The software uses the Needleman-Wunsch alignment algorithm, an automatic procedure for calculating the best possible alignment between two amino acid sequences [27]. This method allows to perform a fast and ingenious comparison between all the alignments among two sequences, considering every possible number of gaps in every possible position. Software's purpose is to choose the best alignment among all produced, simply the one that guarantees the highest 'score'. A short description of the steps used by this tool and an example of the output file are reported in Supplementary Fig. (**1**). Moreover, it is possible to view the entire procedure used by the software at https://www.youtube.com/watch?v=_lm1WOOuv5o&t=67s [28]. First, we performed a protein alignment between the 1273 aa SARS-CoV-2 spike glycoprotein (NCBI Gene ID: NC_045512.2) [14] and the mentioned above Neurotoxin homolog NL1 (UniProtKB - Q9DEQ3 - 3SO8_NAJAT), a polypeptide of 86 aa identified in Najaatra (Chinese cobra) [5, 6]. We noted that the presence of identical or functionally equivalent amino acids is not limited to the 375-390 region, as previously described, but it is also present in other areas of the Spike glycoprotein with a sequence homology of 47/86 aa (54.6%) (Fig. **1**, Supplementary Table **1**). Interestingly, we also found homology for the Muscarinic toxin-like protein, identified in Bungarus multicinctus (Many-banded krait; UniPro

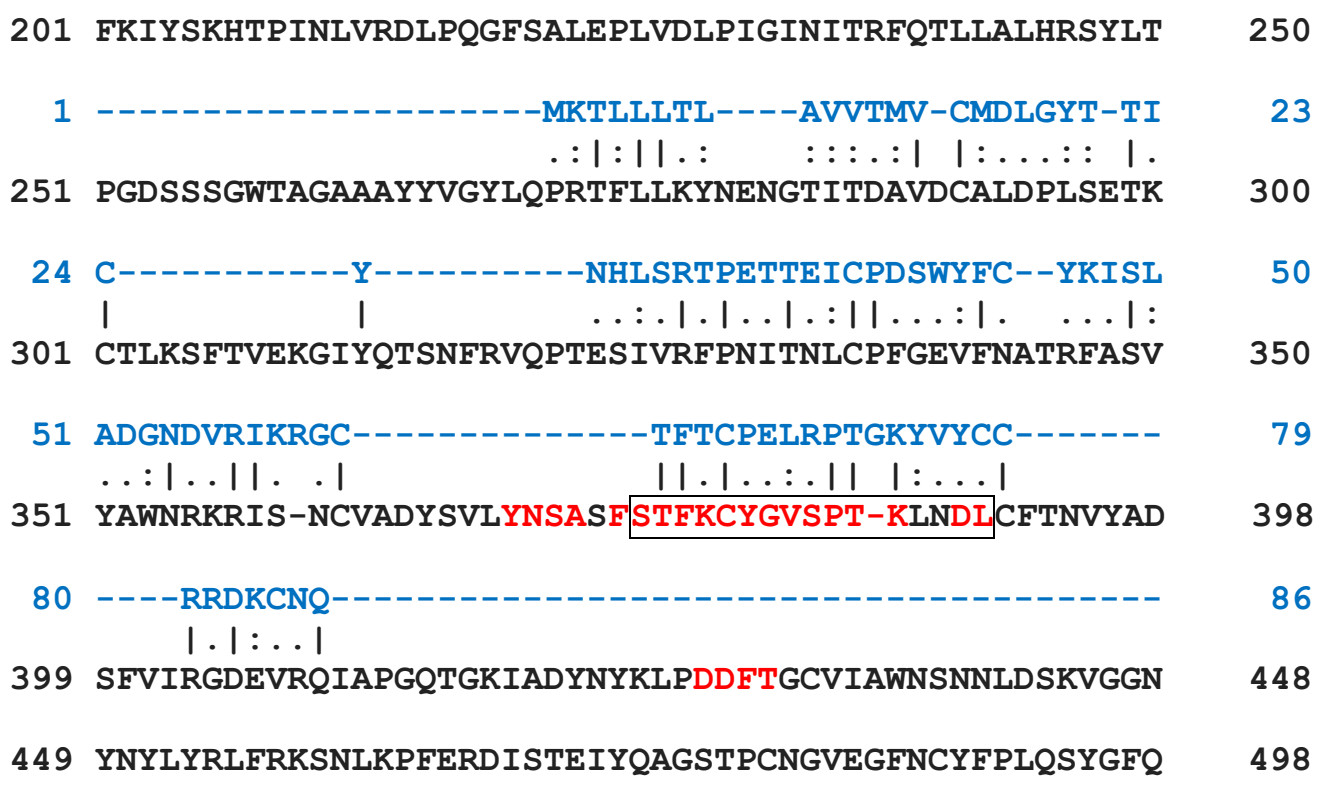

Fig. (1). Protein Sequence Alignment performed using EMBOSS Needle between SARS-CoV-2 Spike glycoprotein (GenBank: NC_045512.2) and Neurotoxin homolog NL1, Najaatra (Chinese cobra) (UniProtKB - Q9DEQ3 - 3SO8_NAJAT) protein. Black: Spike protein. Blue: Conotoxin proteins. Red: SARS-CoV-2 RBD epitope sequence for the CR3022 mAb. Framework: highly conserved region of SARS-CoVs' RBD (375–395 aa). (|): identities, (:) conservative replacements, (.): non-conservative replacements. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

tKB: Q9W727 - 3SO8_BUNMU), which exhibits an entirely similar sequence to Neurotoxin homolog NL1. Furthermore, we found a homology between the cryptic epitope for the human antibody CR3022 and the 27 aa Kappa-conotoxin-like as14a, identified in Conus cancellatus (Cancellate cone; Conus austini; UniProtKB: P0C6S2 - CLEA_CONCF), with an overall sequence homology of 12/27 aa (44.4%) (Supplementary Table **1**). Later, we proceeded to find several conotoxins protein sequences from the UniProt Knowledgebase (UniProtKB) database (https://www.uniprot.org/uniprot/) [29] and always carrying out alignment with SARS-CoV-2 Spike glycoprotein. In all cases, we found a certain rate of homology with the Spike glycoprotein also in regions different from the cryptic epitope for the human antibody CR3022 and with percentages of homology ranging from 22.7% to 50.8%, with an average of 37% (Supplementary Table **1**).

Our *in-silico* analysis, through sequence retrieval and protein alignment, shows high positive correlation between small genome regions and different toxins, like conotoxin, that selectively could interact with nAChRs. Being nAChR antagonists, these toxins are used by cone snails to immobilize their prey [30]. The release of (oligo-) peptides almost identical to animal conotoxins could result in clinical manifestations such as neurological, hemorrhagic and thrombotic. This expression would explain the presence of symptoms of COVID-19 pathogenesis not yet fully clarified, such as prolonged apnea, cardiocirculatory collapse, heart arrhythmias, ventricular tachycardia, body temperature alteration, electrolyte imbalance (particularly K^+) and even the significant reduction of butyrylcholinesterase (BuChE) plasma levels.

Thus, the data obtained from our analyses indicate that regions of homology with neurotoxin-like peptides are more numerous and more widely distributed on the Spike glycoprotein than previously observed by others [31].

3. DISCUSSION

Based on these high levels of homology, we hypothesize that inside the last ORF of viral genome encoding for Spike glycoprotein, the RNA polymerase can encode these small proteins. This hypothesis is supported by several pieces of evidence reporting that SARS-CoV-2 viral RNA polymerase, through a "jumping mechanism" already demonstrated in the other coronavirus, results in numerous discontinuous transcription events, which are likely to include small neurotoxin-like proteins such as conotoxins [32, 33]. Indeed,

while the role of many structural SARS-CoV-2 proteins is well described in the literature, several sgRNAs encoded by RNA polymerase jumping unusual display structure and a still unknown function [34]. The inhibition of AChRs can play a central role in cholinergic CNS and PNS synapses [11] and particularly the AChRs:nicotine binding might block nAChRs, supporting the hypothesis of a protective role for nicotine and other cholinergic agonists, in line with the observation that smokers are almost "protected" against SARS-CoV-2 hospitalization [17, 19, 35]. The absence of nAChRs:nicotine binding could provide logical explanations for acute inflammatory disorder in these patients, as COVID-19 pathology may be linked to severe dysregulation of CNS [10]. Finally, this hypothesis could explain the detection of some toxic products into bloodstream and tissues [36] and the correlation between different taxa compositions of nasopharyngeal or gastrointestinal microbiota and the severity of COVID-19 disease [37].

Certainly, the hypothesis of synthesis of small neurotoxic peptide herein described opens a new scenario on the etiology of COVID-19 clinical symptoms observed so far, including the neurological manifestations. Indeed, the presence of conotoxins-like peptides could explain the appearance of many symptoms including hyposmia, hypogeusia and the typical signs of Guillain Barre syndrome observed in some patients with COVID-19 [38]. As stated, the presence of toxic peptides can alter the normal functioning of ion channels, nicotinic AChR as well as ACh levels and induce a significant reduction of plasmatic BuChE levels [39].

However, the reason for production of these oligopeptides binding to nAChR still remains poorly understood and many doubts remain about the mechanisms related to transcriptional processes of the virus [33, 40]. The above-mentioned literature on computational modeling suggested that SARS-CoV-2 spike glycoprotein might bind to nAChRs by a particular and unusual folding, also through a cryptic epitope that coincides with the well-described cryptic epitope for the human SARS-CoV antibody CR3022 [5, 6, 8, 11]. By the way, it is well known that these peptides are functional macromolecules characterized by a specific 3D "native" structure that allows their functions correctly, once at final conformation [41]. The reaching of the final 3D-folding stability is assisted by specific protein complexes (chaperones) that guarantee the conservation of protein functions [41, 42]. Therefore, it is strongly unlike that Spike protein, specifically a 1273 aa large protein, can totally change its folding to express a small peptide portion of 30 aa able to bind the nicotinic receptor [6]. We strongly support the hypothesis that, through a "jumping mechanism" [33, 40], the RNA polymerase can prime the transcription of small peptides, like conotoxins, whose sequence is contained in the ORF region encoding for Spike glycoprotein. This possibility could be primed by external biological factors such as the host's microbiota [43]. Indeed, recent studies identified a different microbiome depending on ageing and their tissue distribution (gut, lung and eye), allowing us to hypothesize that microbiota can play a crucial role in directing the expression of this small protein by SARS-CoV-2 [32, 43-45]. As observed in COVID-19, elderly subjects are affected by more severe forms of disease, and most probably COVID-19 disease worsens in youngers in the presence of dysbiosis and/or when certain bacterial taxonomies prevail [46-50]. In this context, it is interesting the hypothesis recently proposed that bacterial lipopolysaccharides (LPS) may repress sirt 1 with a consequent effect on nACHRs resulting in a greater severity of covid-19 infection in elderly individuals [24].

Therefore, the microbiome, belonging to specific body districts, represents a valuable "new entry" in the biomedical and therapeutical fields for COVID-19 patients [40, 46, 50].

CONCLUSION

In view of the above reported considerations, some therapeutic solutions can be prospected. First, we must consider that conus venom is highly toxic and lethal as it is composed of many different types of conotoxins (α, δ, κ, μ and ω) which have neurological effects, due to different receptor targets [51, 52]. Indeed, the conotoxins are generally weakly immunogenic and therefore, not effectively targeted by current polyclonal anti-venom therapies [53]. Although the use of monoclonal anti-toxin directed either towards the virus surface antigens and/or the released virus products could be a quick solution to reduce mortality rates, it remains difficult to apply. On the contrary, nAChR can bind these toxins and therefore, it can be used against neurotoxic envenoming [53-56]. The administration of cholinesterase-derived human BuChE could be hypothesized as a potential therapeutic approach [51]. On the other hand, a therapeutic approach using nAChRs agonists, as hypothesized by a previous *in silico* study, cannot be ruled out [10].

Undoubtedly, our hypothesis remains to be proven and numerous efforts still need to be made by researchers to eradicate this serious pandemic that is affecting health and the economy globally.

LIST OF ABBREVIATIONS

hACE2	=	human Angiotensin-Converting Enzyme 2
CNS	=	central Nervous System
nAChRs	=	nicotinic Acetyl-Choline Receptors
ORF	=	Open Reading Frame
BuChE	=	butyryl Cholinesterase
WHO	=	World Health Organization
RBD	=	Receptor-Binding Domain
IL-1	=	interleukin-1
IL-6	=	interleukin-6
IL-8	=	interleukin-8
SIRT1	=	Sirtuin 1
TNF-α	=	Tumor Necrosis Factor-α
EMBL-EBI	=	European Molecular Biology Laboratory - European Bioinformatics Institute

EMBOSS = European Molecular Biology Open Software Suite

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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