

1 **Alzheimer's-like remodeling of neuronal ryanodine receptor in COVID-19**

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24 **Summary**

25 **COVID-19, caused by SARS-CoV-2 involves multiple organs including cardiovascular,**
26 **pulmonary and central nervous system. Understanding how SARS-CoV-2 infection afflicts**
27 **diverse organ systems remains challenging^{1,2}. Particularly vexing has been the problem**
28 **posed by persistent organ dysfunction known as “long COVID,” which includes cognitive**
29 **impairment³. Here we provide evidence linking SARS-CoV-2 infection to activation of**
30 **TGF- β signaling and oxidative overload. One consequence is oxidation of the ryanodine**
31 **receptor/calcium (Ca^{2+}) release channels (RyR) on the endo/sarcoplasmic (ER/SR) reticuli**
32 **in heart, lung and brains of patients who succumbed to COVID-19. This depletes the**
33 **channels of the stabilizing subunit calstabin2 causing them to leak Ca^{2+} which can promote**
34 **heart failure^{4,5}, pulmonary insufficiency⁶ and cognitive and behavioral defects⁷⁻⁹. *Ex-vivo***
35 **treatment of heart, lung, and brain tissues from COVID-19 patients using a Rycal drug**
36 **(ARM210)¹⁰ prevented calstabin2 loss and fixed the channel leak. Of particular interest is**
37 **that neuropathological pathways activated downstream of leaky RyR2 channels in**
38 **Alzheimer’s Disease (AD) patients⁷ were activated in COVID-19 patients. Thus, leaky**
39 **RyR2 Ca^{2+} channels may play a role in COVID-19 pathophysiology and could be a**
40 **therapeutic target for amelioration of some comorbidities associated with SARS-CoV-2**
41 **infection.**

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43 **Key words: COVID-19, TGF- β , SMAD3, NOX2, RyR2, Rycal.**

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48 **Main**

49 Patients suffering from COVID-19 exhibit multi-organ system failure involving not only
50 pulmonary ¹ but also cardiovascular ², neural ¹¹ and other systems. The pleiotropy and
51 complexity of the organ system failures both complicate the care of COVID-19 patients, and
52 contribute to a great extent to the morbidity and mortality of the pandemic¹². Clinical data¹³
53 indicate that severe COVID-19 most commonly manifests as viral pneumonia-induced acute
54 respiratory distress syndrome (ARDS). Respiratory failure results from severe inflammation in
55 the lungs, which arises when COVID-19 infects lung cells and damages them. Cardiac
56 manifestations are multifactorial, and include hypoxia, hypotension, enhanced inflammatory
57 status, angiotensin-converting enzyme 2 (ACE2) receptor downregulation, endogenous
58 catecholamine adrenergic status, and direct viral myocardial damage^{14,15}. Moreover, patients
59 with underlying cardiovascular disease or comorbidities, including congestive heart failure,
60 hypertension, diabetes, and pulmonary diseases, are more susceptible to infection by SARS-
61 CoV-2, with higher mortality^{14,15}. In addition to respiratory and cardiac manifestation, it has been
62 reported that 36.4% of patients with COVID-19 develop neurological symptoms, including
63 headache, disturbed consciousness, and paresthesia ¹⁶. Brain tissue edema, stroke, partial
64 neuronal degeneration in deceased patients, and neuronal encephalitis have also been reported^{2,16-}
65 ¹⁸. Furthermore, another pair of frequent symptoms of infection by SARS-CoV-2 are hyposmia
66 and hypogeusia, the loss of the ability to smell and taste, respectively^{11,19}. Interestingly,
67 hyposmia has been reported in early stage AD¹¹ and Alzheimer type II astrocytosis has been
68 observed in neuropathology studies of COVID-19 patients¹⁸. Despite the myriad of neurological

69 symptoms, manifestations, and complications reported with SARS-CoV-2 infection^{20,21}, a
70 systematic study of COVID-19 brain neuropathology is still needed²².

71 Systemic failure in COVID-19 patients is likely due to SARS-CoV-2 invasion via the
72 ACE2 receptor¹⁷, which is highly expressed in pericytes of human heart²³, epithelial cells of the
73 respiratory tract²⁴, kidney, intestine, and blood vessels. ACE2 is also expressed in the brain,
74 especially in the brainstem, specifically the respiratory center and hypothalamus, the thermal
75 center, and cortex²⁵, which renders these tissues more vulnerable to viral invasion. The primary
76 consequences of SARS-CoV-2 infection are inflammatory responses and oxidative stress in
77 multiple organs and tissues²⁶⁻²⁸. Recently it has been shown that the high neutrophil to
78 lymphocyte ratio observed in critically ill patients with COVID-19 is associated with excessive
79 levels of reactive oxygen species (ROS) and that ROS induced tissue damage, contributing to
80 COVID-19 disease severity²⁶. The impact of oxidative stress on calcium (Ca^{2+}) homeostasis, a
81 key determinant of cardiac function and rhythm²⁹, as well as neuronal activity, remain to be
82 elucidated.

83 Recent studies have explored the relationship between ACE2 and TGF- β , demonstrating
84 the inverse relationship between the two. In cancer models, decreased levels of ACE2 correlated
85 with increased levels of TGF- β ³⁰. In the context of SARS-CoV-2 infection, downregulation of
86 ACE2 has been observed, leading to increased fibrosis formation, as well as upregulation of
87 TGF- β and other inflammatory pathways³¹. Finally, patients with severe COVID-19 symptoms
88 had higher blood serum TGF- β concentrations than those with mild symptoms³², thus further
89 implicating the role of TGF- β in this disease and warranting further investigation on the topic.

90 Downregulation of ACE2 has been observed in SARS-CoV-2 infection, leading to
91 increased fibrosis formation, as well as upregulation of TGF- β and other inflammatory

92 pathways³¹. Interestingly, reduced angiotensin/ACE-2 activity has been associated with Tau
93 hyper-phosphorylation and increased amyloid- β pathology in animal models of Alzheimer
94 disease^{33,34}. The link between reduced ACE2 activity and increased TGF- β and Tau signaling in
95 the context of SARS-CoV-2 infection needs further exploration.

96 Our laboratory has shown that stress-induced ryanodine receptor (RyR)/intracellular
97 calcium release channel post-translational modifications, including oxidation and protein kinase
98 A (PKA) hyper-phosphorylation related to activation of the sympathetic nervous system and the
99 resulting hyper-adrenergic state, deplete the channel stabilizing protein (calstabin) from the
100 channel complex, destabilizing the closed state of the channel causing it to leak in multiple
101 diseases^{6-9,29,35,36}. Increased transforming growth factor- β (TGF- β) activity can lead to RyR
102 modification and leaky channels³⁷ and SR Ca²⁺ leak can cause mitochondrial Ca²⁺ overload and
103 dysfunction³⁶. Increased TGF- β activity³⁸ and mitochondrial dysfunction³⁹ are also associated
104 with SARS-CoV-2 infection.

105 Here we show that SARS-CoV-2 infection is associated with oxidative stress and
106 activation of the TGF- β signaling pathway in heart, lung and brain of patients who have
107 succumbed to COVID-19. One consequence is RyR oxidation, particularly through NADPH
108 oxidase 2 (NOX2), rendering the channels leaky to Ca²⁺ which may play a role in the
109 pathological manifestations of SARS-CoV-2 infection in heart, lung and brain. Moreover, the
110 hyper-adrenergic state observed in COVID-19 patients causes PKA hyper-phosphorylation of
111 RyR2 on Serine 2808, loss of the stabilizing subunit calstabin2 from the channel complex and
112 leaky RyR2 channels in heart, lung, and brain. We also demonstrate that SARS-CoV-2 infection
113 activates biochemical pathways linked to the Tau pathology associated with AD and that leaky

114 calcium channels may be a potential therapeutic target for the respiratory, cardiac and neuronal
115 complications associated with COVID-19.

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117 **Results**

118 **Oxidative stress and TGF- β activation**

119 Oxidative stress was determined in heart, lung, and brain tissues from COVID-19 patient
120 autopsy tissues and controls by measuring the ratio of glutathione disulfide (GSSG) to
121 glutathione (GSH). COVID-19 patients exhibited significant oxidative stress with a 7.7, 13.6,
122 and 3.2-fold increase in GSSG/GSH ratios in heart, lung, and brain compared to controls
123 respectively (**Figure 1A**). In order to determine if SARS-CoV-2 infection also increases tissue
124 TGF- β activity, we measured SMAD3 phosphorylation, a downstream signal of TGF β , in control
125 and COVID-19 tissue lysates (**Figure 1B**). Phosphorylated SMAD3 (pSMAD3) levels were
126 increased in COVID-19 heart, lung, and brain lysates compared to controls, indicating that
127 SARS-CoV-2 infection increased TGF- β signaling in these tissues. Interestingly, brain tissues
128 from COVID-19 patients exhibited activation of the TGF- β pathway, despite the absence of the
129 detectable (by immunohistochemistry and PCR, data not shown) virus in these tissues. These
130 results suggest that the TGF- β pathway is activated systemically by SARS-CoV-2 resulting in its
131 upregulation in brain as well as other organs.

132 **RyR2 channel oxidation and leak**

133 RyR channels may be oxidized as a consequence of activation of the TGF- β signaling
134 pathway³⁷. NOX2 binding to RyR2 causes oxidation of the channel that activates the channel
135 manifested as an increased open probability. When the oxidization of the channel is at pathologic

136 levels there is destabilization of the closed state of the channel resulting in spontaneous Ca^{2+}
137 release or leak^{35,40}. To determine the effect of the increased TGF- β signaling associated with
138 SARS-Cov-2 infection on NOX2/RyR2 interaction, RyR2 and NOX2 were co-
139 immunoprecipitated from heart, lung, and brain lysates of COVID-19 patients and controls.
140 NOX2 associated with RyR2 in heart, lung, and brain tissues from SARS-CoV-2 infected
141 individuals was increased compared to controls (**Figure 1C**).

142 The SMAD3 proteins involved in TGF- β signaling were shown to interact with the
143 nucleocapsid (N) protein in SARS-CoV1³⁸, interfering with healthy apoptotic pathways. As a
144 result, apoptosis of SARS-CoV-2 infected host cells is blocked and formation of tissue fibrosis is
145 promoted, especially in lung, thus contributing to the respiratory distress and subsequent
146 pulmonary failure associated with the disease. SARS-CoV-2 infection may also activate TGF- β
147 signaling through a direct interaction of the viral nucleocapsid protein (N protein) with SMAD3.
148 This direct interaction is shown in **Figure 1D**, which demonstrates that SMAD3 and SARS-
149 CoV-2 N protein co-immunoprecipitated from COVID-19 heart and lung lysates using
150 antibodies specific for either protein. The brain lysates used in this study did not contain
151 detectable (by immunoprecipitation/immunoblotting) SARS-CoV-2 N protein, indicating that
152 indirect effects of the inflammatory/oxidative response may be responsible for the alterations in
153 brain biochemistry observed in **Figure 1**.

154 Given the increased oxidative stress and increased NOX2 binding to RyR2 seen in
155 COVID-19 tissues, RyR2 post-translational modifications in these tissues were investigated.
156 Immunoprecipitated RyR2 from heart, lung, and brain lysates demonstrated increased oxidation,
157 PKA phosphorylation on Serine 2808, and depletion of the stabilizing protein subunit calstabin2
158 in SARS-CoV-2 infected tissues compared to controls (**Figure 2A-C**).

159 RyR channel activity was determined by binding of $^3\text{[H]}$ ryanodine, which binds only to
160 the open state of the channel. RyR2 was immunoprecipitated from tissue lysates and ryanodine
161 binding to the immunoprecipitated was measured at both 150 nM and 20 μM free Ca^{2+} . The total
162 amount of RyR immunoprecipitated was the same for control and COVID-19 samples
163 (immunoblots in Figure 2D). However, RyR2 channels from SARS-CoV-2 infected heart, lung,
164 and brain tissue demonstrate abnormally high activity compared to channels from control tissues
165 at physiologically resting conditions (150 nM free Ca^{2+}), when channels should be closed (Figure
166 2D). This biochemical/functional remodeling of the channel results in what is known as “the
167 biochemical signature” of leaky RyR2^{4,41} that destabilizes the closed state of the channel. This
168 leads to SR/ER chronic Ca^{2+} leak which contributes to the pathophysiology of various diseases⁴⁻
169 ^{7,9,37,42}. Rebinding of calstabin2 to RyR2, using a Rycal, has been shown to reduce SR/ER Ca^{2+}
170 leak, despite the persistence of the channel remodeling. Indeed, calstabin2 binding to RyR2 was
171 increased when COVID-19 patient heart, lung and brain tissue lysates were treated *ex-vivo* with
172 the Rycal drug ARM210 (Figure 2A-C). RyR2 activity at resting Ca^{2+} concentration was also
173 decreased by Rycal treatment (Figure 2D).

174 **Activation of Alzheimer’s Disease linked signaling**

175 Leaky ryanodine receptors have recently been implicated in the neurodegenerative
176 processes that contribute to the etiology of Alzheimer's and Huntington’s disease^{7,8}. Abnormal
177 Ca^{2+} handling can contribute to mitochondrial Ca^{2+} overload, dysregulation of Ca^{2+} -dependent
178 enzymes such as AMP-activated protein kinase (AMPK), cyclin-dependent kinase 5 (CDK5),
179 and enhanced calpain activity⁷. Activation of these enzymes in response to elevated cytosolic
180 Ca^{2+} levels is upstream of both Tau and amyloid deposits in Alzheimer’s disease brains, and
181 could play an important role in the “brain fog” associated with COVID-19. Brain lysates from

182 COVID-19 patients' autopsies demonstrated increased AMPK and GSK3 β phosphorylation
183 compared to controls (**Figure 3A-B**). Activation of these kinases in SARS-CoV-2 infected brain
184 leads to a hyper-phosphorylation of Tau similar to that observed in Alzheimer Tau pathology.
185 COVID-19 brain lysates showed increased Tau phosphorylation at S199 and S202/T205 (**Figure**
186 **3A-B**). We also observed an increased p25 expression, the neurotoxic activator of CDK5
187 (**Figure 3C-D**). CDK5 plays an important role in amyloid precursor protein (APP) processing in
188 AD. However, the increased p25/CDK5 observed in COVID-19 brain lysates did not activate
189 the amyloid beta pathway as no differences observed in BACE1 or BCTF levels between control
190 and COVID-19 brain lysates (**Figure 3C-D**).

191 **Discussion**

192 The molecular basis of how SARS-CoV-2 infection affects various tissues is not well
193 understood, and questions regarding the role of defective Ca²⁺ signaling in heart, lung, and brain
194 remain unanswered. In this study, we propose a potential mechanism that may contribute to
195 systemic organ failure caused by SARS-CoV-2: defective Ca²⁺ regulation and its downstream
196 signaling.

197 TGF- β belongs to a family of cytokines involved in the formation of cellular fibrosis by
198 promoting epithelial-to-mesenchymal transition, fibroblast proliferation, and differentiation⁴³.
199 TGF- β activation has been shown to induce fibrosis in lung and other organs by activation of the
200 SMAD-dependent pathway. We have previously reported that TGF- β /SMAD3 activation leads to
201 NOX2/4 translocation to the cytosol and association with RyR channels promoting oxidization of
202 the channels and depletion of the stabilizing subunit calstabin in skeletal muscle and in heart^{35,37}.
203 Alteration of Ca²⁺ signaling may be particularly crucial in COVID-19 infected patients with
204 cardiovascular/neurological diseases due, in part, to the multifactorial RyR2 remodeling

205 following the cytokine storm, increased TGF- β activation, and increased oxidative stress.
206 Moreover, SARS-CoV-2 infected patients exhibited increased endogenous adrenergic state,
207 which leads to hyper-phosphorylation of RyR2 channels, as observed in this study, promoting
208 pathological remodeling of the channel and exacerbating defective Ca²⁺ regulation that likely
209 result in cardiomyopathies and arrhythmias. In line with our observation, a recent study has
210 reported evidence of takotsubo cardiomyopathy caused by a stroke and an acute COVID-19
211 induced sympathetic stimulation and catecholamines surge which has led to death⁴⁴.

212 **Conclusion**

213 Our data indicate a role for leaky RyR2 in the pathophysiology of SARS-CoV-2 infection
214 (**Figure 4**). We show increased systemic oxidative stress and activation of the TGF- β signaling
215 pathway in lung, heart and brain of COVID-19 patients that correlates with oxidation-driven
216 biochemical remodeling of the ryanodine receptor calcium release channel (RyR2). This RyR2
217 remodeling results in intracellular calcium leak which can promote heart failure, pulmonary
218 insufficiency and cognitive dysfunction. Of particular interest is that leaky RyR2 channels in the
219 brain were associated with activation of neuropathological pathways that are also found in the
220 brains of Alzheimer's Disease patients. *Ex-vivo* treatment of COVID-19 lung, heart and brain
221 using a Rycal drug (ARM210) that targets RyR2 channels prevented intracellular Ca²⁺ leak in
222 patient samples.

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226 **Author contributions:** HD, LS, SR, XL and ARM designed experiments, analyzed data and
227 edited/wrote the paper.

228 **Competing interests:** Columbia University and ARM own stock in ARMGO, Inc. a company
229 developing compounds targeting RyR and have patents on Rycals

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232 **Data availability**

233 The data supporting the findings of this are documented within the paper and are available from
234 the corresponding author upon request.

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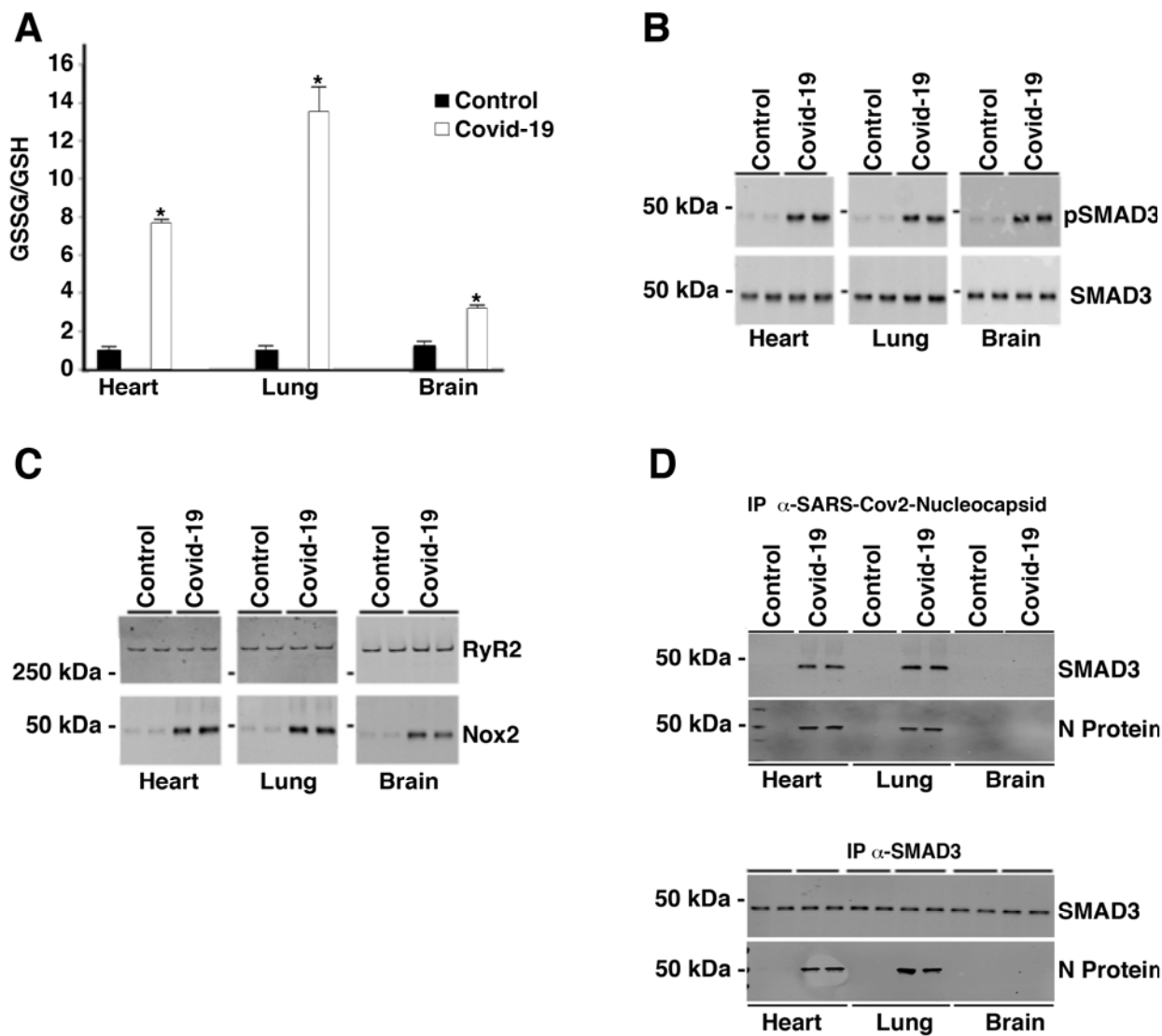
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355 **Figures and figure legends:**



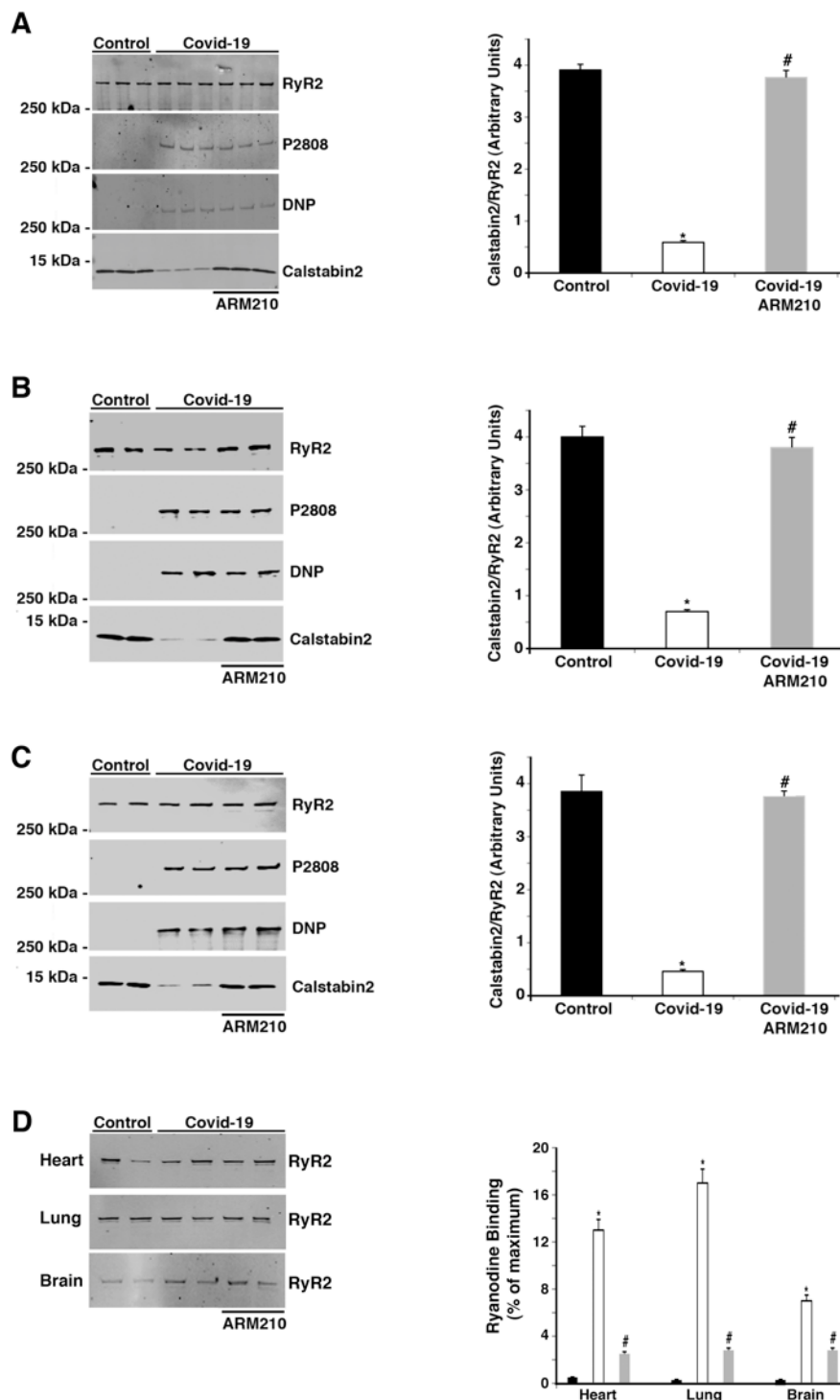
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358 **Figure 1- Increased oxidative stress in heart, lung, and brain of COVID-19 patients.**

359 (A) Bar graph depicting the GSSG/GSH ratio from control (n=2) and COVID-19 (n=2) tissue lysates.
 360 *p<0.05 control vs COVID-19. (B) Western blots showing Phospho-SMAD3 and total SMAD3 from
 361 control (n=2) and COVID-19 (n=2) tissue lysates. (C). Western blots showing the co-
 362 immunoprecipitation of NOX2 with RyR2 from control (n=2) and COVID-19 (n=2) tissue lysates. (D).
 363 Western blots showing the co-immunoprecipitation of SARS-CoV-2-nucleocapsid (N protein) with
 364 SMAD3. Upper panel are westerns of immunoblots blotted for both N protein and SMAD3 from lysates
 365 that were immunoprecipitated with anti-SARS-CoV-2-nucleocapsid antibody. Bottom panel are westerns
 366 of immunoblots blotted for both N protein and SMAD3 from lysates that were immunoprecipitated with
 367 anti-SMAD3 antibody.

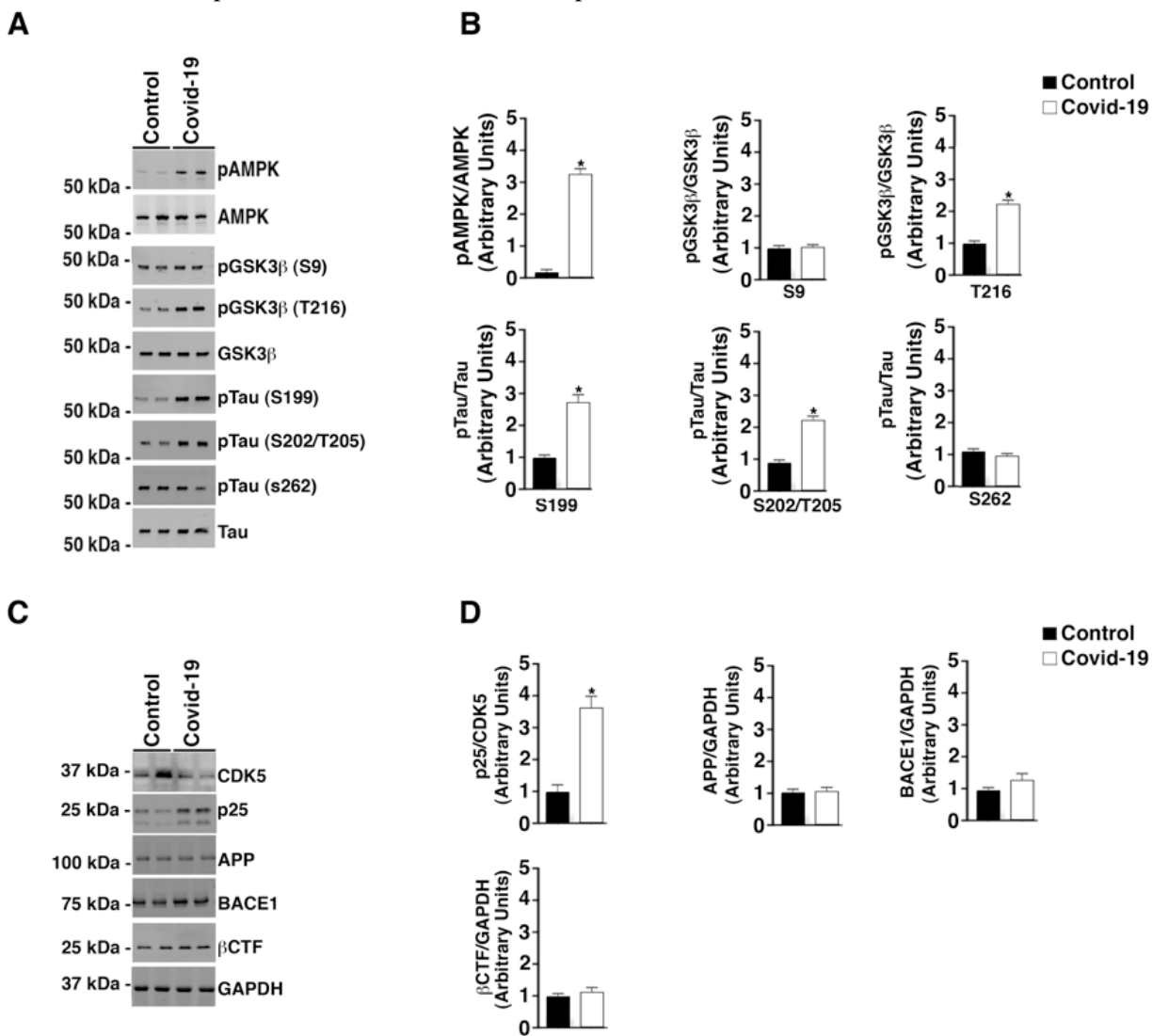
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370 **Figure 2 - RyR2 post-translational modifications and calstabin2 binding in COVID-19 tissues. (A)**
371 **Western blots (left panel) and quantification of calstabin2/RyR2 association from heart lysates (right**
372 **panel). (B) Western blots (left panel) and quantification of calstabin2/RyR2 association from lung lysates**
373 **(right panel). (C) Western blots (left panel) and quantification of calstabin2/RyR2 association from brain**
374 **lysates (right panel). (D) ³[H]ryanodine binding from immunoprecipitated RyR2. Bar graphs show**

375 ryanodine binding at 150 nM Ca^{2+} as a percent of maximum binding ($\text{Ca}^{2+} = 20 \mu\text{M}$). Data are mean \pm
 376 SD. t-test revealed * $p < 0.05$ control vs COVID-19; # $p < 0.05$ COVID-19 vs COVID-19+ARM210.

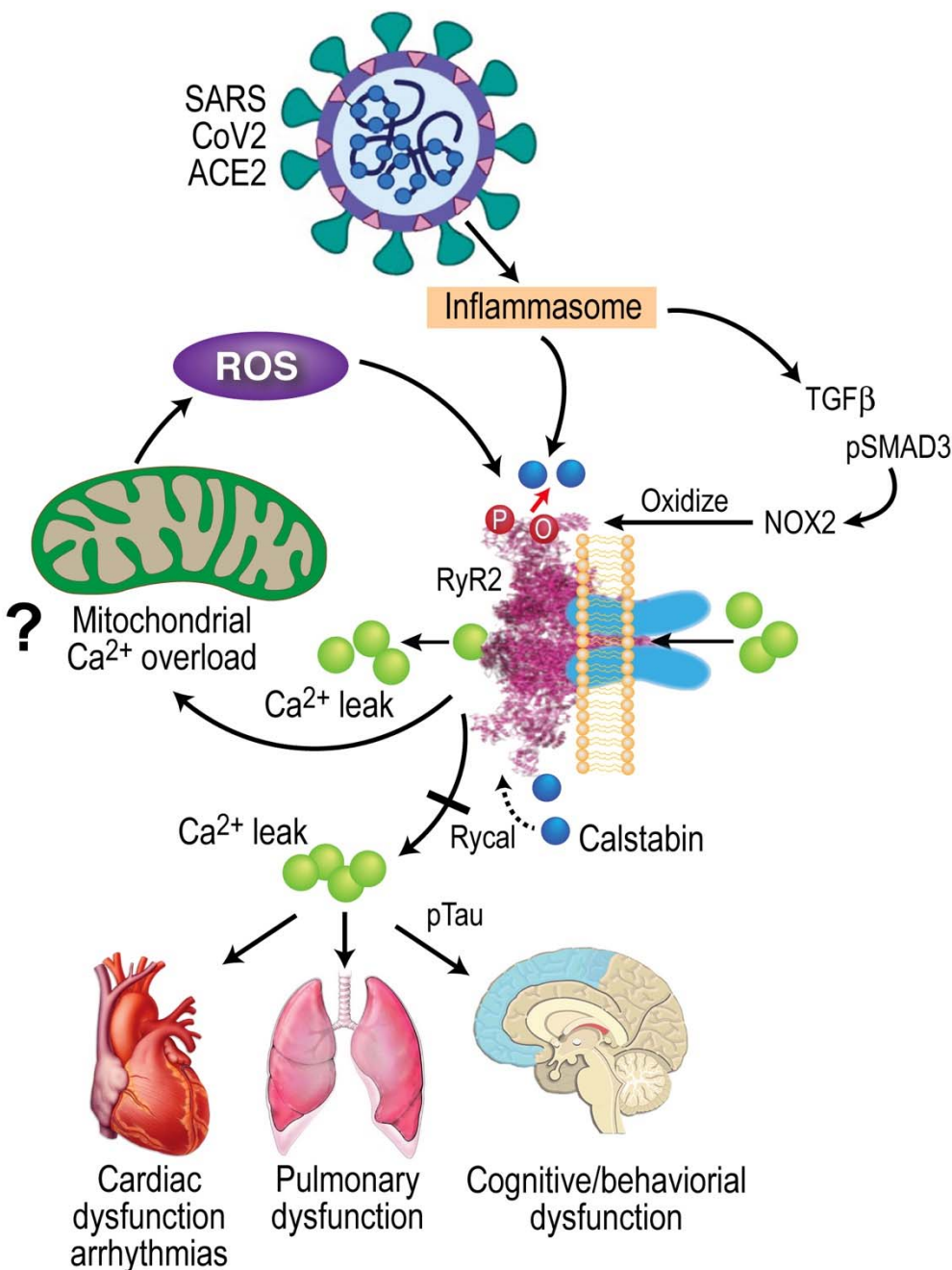


377

378 **Figure 3. Hyperphosphorylation of Tau but normal APP processing in COVID-19 brain lysates.** (A)
 379 Brain lysates were separated by 4-20% PAGE. Immunoblots were developed for pAMPK, AMPK,
 380 GSK3 β , pGSK3 β (S9, T216), Tau, pTau (S199, S202/T205, S262). (B) Bar graphs showing
 381 quantification of phosphorylated AMPK, pGSK3 β and pTau from westerns. (C). immunoblots developed
 382 for CDK5, CDK5 truncated activator p25, APP, BACE1, and β CTF. (D) Bar graphs showing
 383 quantification p25/CDK5 and expression of APP, BACE1, and β CTF compared to loading control
 384 (GAPDH). Data are mean \pm SD. t-test revealed * $p < 0.05$ control vs COVID-19.

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388 **Figure 4. SARS-CoV-2 infection results in leaky RyR2 that may contribute to cardiac, pulmonary,**
389 **and cognitive dysfunction.** SARS-CoV-2 infection targets cells via the ACE2 receptor inducing
390 inflammasome stress response/activation of stress signaling pathways. This results in increased TGF-β
391 signaling, which activates SMAD3 (phosphoSMAD3, pSMAD), this increases NOX2 expression and the
392 amount of NOX2 associated with RyR2. Increased NOX2 activity at RyR2 oxidizes the channel causing
393 calstabin depletion from the channel macromolecular complex, destabilization of the closed state, and
394 ER/SR calcium leak that is known to contribute to cardiac dysfunction^{4,5}, arrhythmias²⁸, pulmonary
395 insufficiency^{6,8}, and cognitive and behavioral abnormalities associated with neurodegeneration^{7,8}. Rycal
396 drugs fix the RyR channel leak by restoring calstabin binding and stabilizing the channel closed state.
397 Fixing leaky RyR may improve cardiac, pulmonary and neural function in COVID-19.

398 **Methods**

399 **Human samples**

400 De-identified human heart, lung, and brain tissue from the Covid BioBank at Columbia
401 University. The Columbia University biobank functions under standard operating procedures,
402 quality assurance, and quality control for sample collection and maintenance. Age and gender-
403 matched controls exhibited absence of neurological disorders and cardiovascular or pulmonary
404 diseases.

406 **Lysate preparation and western blots**

407 Tissue (50 mg) were isotonicallly lysed using a dounce homogenizer in 0.25 ml of 10 mM Tris
408 Maleate (pH 7.0) buffer plus protease inhibitors (Complete inhibitors from Roche). Samples
409 were centrifuged at 8,000 x g and the protein concentration of the supernatants were determined
410 by Bradford assay. To determine protein levels in tissue lysates, tissue proteins (20 µg) were
411 separated by 4-20% SDS-PAGE and immunoblots were developed using antibodies against
412 pSMAD3 (Abcam, 1:1000), SMAD3 (Abcam, 1:1000), AMPK (Abcam, 1:1000), pAMPK
413 (Abcam, 1:1000), CDK5 (Thermofisher, 1: 1,000), and p25 (Thermofisher, 1:1000). Tau
414 (Thermofisher, 1:1000), P-Tau (S199, Thermofisher, 1:1000), P-Tau (S202/T205, Abcam, P-Tau
415 (S262, Abcam, 1:1000). 1:1000), GSK3β (Abcam, 1: 2,000)), p- GSK3β (S9, Abcam, 1: 2,000),
416 p- GSK3β (T216, Abcam, 1: 2,000), APP (Abcam, 1: 2,000), BACE1 (Abcam, 1: 2,000),
417 GAPDH (Santa Cruz Bioteck, 1:1000), βCTF (Abcam, 1: 2,000), SARS Cov-2 nucleocapsid
418 (Thermofisher, 1: 1,000).

419 **Analysis of ryanodine receptor complex**

420 Tissue lysates (0.1 mg) were treated with buffer or 10 μ M Rycal (ARM210) at 4°C. RyR2 were
421 immunoprecipitated from 0.1 mg lung, heart and brain using an anti-RyR2 specific antibody (2
422 μ g) in 0.5 ml of a modified radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.2,
423 0.9% NaCl, 5.0 mM NaF, 1.0 mM Na₃VO₄, 1% Triton X-100, and protease inhibitors) overnight
424 at 4 °C. RyR2 specific antibody was an affinity-purified polyclonal rabbit antibody using the
425 peptide CKPEFNNHKDYAQEK corresponding to amino acids 1367-1380 of mouse RyR2 with
426 a cysteine residue added to the amino terminus. The immune complexes were incubated with
427 protein A-Sepharose beads (Sigma) at 4 °C for 1 h, and the beads were washed three times with
428 radioimmune precipitation assay buffer. The immunoprecipitates were size-fractionated on SDS-
429 PAGE gels (4-20 % for RyR2, calstabin and NOX2) and transferred onto nitrocellulose
430 membranes for 2 h at 200 mA. Immunoblots were developed using the following primary
431 antibodies: anti-RyR2 (Affinity Bioreagents, 1:2000), anti-phospho-RyR-Ser(P)-2808 (Affinity
432 Bioreagents 1:5000), anti-calstabin (FKBP12 C-19, 1:1000, Santa Cruz Biotechnology, Inc.,
433 Santa Cruz, CA), anti NOX2 (Abcam, 1:1000). To determine channel oxidation, the carbonyl
434 groups in the protein side chains were derivatized to DNP by reaction with 2,4-
435 dinitrophenylhydrazine. The DNP signal associated with RyR was determined using a specific
436 anti-DNP antibody according to the manufacturer's instructions (Millipore, Billerica, MA). All
437 immunoblots were developed and quantified using an Odyssey system (LI-COR Biosciences,
438 Lincoln, NE) with IR-labeled anti-mouse and anti-rabbit IgG (1: 10,000 dilution) secondary
439 antibodies.

440 **Ryanodine Binding**

441 RyR2 were immunoprecipitated from 1.5 mg of tissue lysate using an anti-RyR2 specific
442 antibody (25 μ g) in 1.0 ml of a modified RIPA buffer overnight at 4 °C. The immune complexes

443 were incubated with protein A-Sepharose beads (Sigma) at 4 °C for 1 h, and the beads were
444 washed three times with RIPA buffer, followed by 2 washes with ryanodine binding buffer (10
445 mM Tris-HCl, pH 6.8, 1 M NaCl, 1 % CHAPS, 5 mg/ml phosphatidylcholine, and protease
446 inhibitors). Immunoprecipitates were incubated in 0.2 ml of binding buffer containing 20 nM
447 [³H] ryanodine and either of 150 nM and 20 μm free Ca²⁺ for 1 h at 37 °C. Samples were diluted
448 with 1 ml of ice-cold washing buffer (25 mM Hepes, pH 7.1, 0.25 M KCl) and filtered through
449 Whatman GF/B membrane filters pre-soaked with 1% polyethyleneimine in washing buffer.
450 Filters were washed three times with 5ml of washing buffer. The radioactivity remaining on the
451 filters is determined by liquid scintillation counting to obtain bound [³H] ryanodine. Nonspecific
452 binding was determined in the presence of 1000-fold excess of non-labeled ryanodine.

453 **GSSH/GSH ratio measurement and SMAD3 phosphorylation**

454 Approximately 20 mg of suspended in 200 μL of ice-cold PBS/0.5% NP-40, pH6.0 for lysis.
455 Tissue is homogenized with a Dounce homogenizer with 10 – 15 passes. Samples are
456 centrifuged at 8,000 x g for 15 minutes at 4°C to remove any insoluble material. Supernatant is
457 transferred to a clean tube. Deproteinizing of the samples is accomplished by adding 1 volume
458 ice cold 100% (w/v) TCA into 5 volumes of sample and vortexing briefly to mix well. After
459 incubating for 5 min on ice, samples are centrifuged at 12,000 x g for 5 minutes at 4°C and the
460 supernatant is transferred to a fresh tube. The samples are neutralized by adding NaHCO₃ to
461 supernatant and vortexing briefly. Samples are centrifuged at 13,000 x g for 15 minutes at 4°C
462 and supernatant is collected. Samples are now deproteinized, neutralized, and TCA has been
463 removed. The samples are ready to use in the assay. The GSSG/GSH is determined using the
464 ratio detection assay kit (Abcam, ab138881). Briefly, in two separate assay reactions, GSH
465 (reduced) is measured directly with a GSH standard and Total GSH (GSH + GSSG) is measured

466 by using a GSSG standard. A 96 well plate is set up with 50 μ L duplicate samples and standards
467 with known concentrations of GSH and GSSG. A Thiol green indicator is added and the plate is
468 incubated for 60 min at room temperature. Fluorescence at Ex/Em = 490/520 nm is measured
469 with a fluorescence microplate reader, and the GSSG/GSH for samples are determined
470 comparing fluorescence signal of samples with known standards.

471 **Statistics**

472 Group data are presented as mean \pm SEM. Statistical comparisons between the two groups were
473 tested using an unpaired t test. Values of $p < 0.05$ were considered statistically significant. All
474 statistical analyses were performed with Prism 8.0.

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