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Sudan Black B treatment uncovers the distribution of angiotensin-converting enzyme2 in nociceptors

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Abstract

Nervous system manifestations caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are of great concern. Neurological symptoms and the neurological effects induced by SARS-CoV-2, such as the loss of various sensory perceptions, indicate direct viral invasion into sensory neurons. Therefore, it is very important to identify the distribution of angiotensin-converting enzyme 2 (ACE2), the receptor of SARS-CoV-2, in human nervous system. However, autofluorescence from lipofuscin obviously impacted immunofluorescence analysis in previous studies. We demonstrated that Sudan Black B (SBB) remarkably reduced the massive lipofuscin-like autofluorescence and the immunofluorescence signal would be sharpened following the exposure compensation. Additionally, we confirmed that ACE2 was expressed in IB4+, CGRP+, and NF200+ sensory subpopulations. The mapping of ACE2 distribution in hDRG would facilitate the understanding of sensory disorder induced by SARS-CoV-2.

Keywords

COVID-19, ACE2, lipofuscin-like autofluorescence, SBB

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COVID-19 caused by SARS-CoV-2 has raged around the world, which is characterized by pneumonia, respiratory distress, and hypercoagulation. Many extrapulmonary manifestations of COVID-19 have been observed, of which neurological symptoms have driven increasing concern.[1–3] These neurological symptoms are diverse, ranging from the loss of various sensory perceptions to the life-threatening acute disseminated encephalomyelitis.[2] The detailed mechanisms of neurological symptoms in COVID-19 are still not explicit. However, direct viral invasion has been considered one of the key pathogenic pathways, and sensory-specific neurological effects including joint pain, headache, loss of smell, taste and chemesthesis, etc. suggest the involvement of sensory neural system.[1] Many pre-clinical studies have shown direct coronavirus invasion in the nervous tissues and explored the distribution of angiotensin-converting enzyme 2 (ACE2), the receptor for SARS-CoV-2, in the nervous system.[4–6] Meanwhile, relative enrichment of ACE2 expression was detected in nasal epithelial cells from human lung tissue.[7,8] This was consistent with the common hypogeusia and hyposmia symptoms of COVID-19. Therefore, it is very important to further identify the distribution of ACE2 in the human nervous system and many researchers have contributed to this. For example, Shiers et al.[9] have detected ACE2 in human dorsal root ganglion (hDRG) and a subset of *MRGPRD*$^{+}$ nociceptors co-expressed ACE2.[9] However, we noticed that autofluorescence in immunofluorescent staining of human neural tissues was a universal

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problem disturbing the veracity of conclusions. The autofluorescence should not be ignored while studying the distribution of ACE2, and a reliable method should be selected to reduce its impacts.

Immunofluorescence analysis is distinctly affected by biological autofluorescence, which generates in animal tissues with age. Lipofuscin is a major source of autofluorescence in cytoplasm of the nervous system, especially in human CNS. It is effective to some extent to distinguish non-specific background fluorescence from target fluorescence by setting up negative controls. However, the presence of autofluorescence might still disturb the statistical process. It had been illustrated that preprocessing with 1–10 mM $CuSO_4$ in 50 mM ammonium acetate buffer (pH, 5.0) or Sudan Black B (SBB) in 70% ethanol is a practicable method to reduce lipofuscin-like autofluorescence in 1999 by Schnell et al.[10] SBB was also proved to be more efficient to eliminate lipofuscin-like autofluorescence in aged human CNS tissue. Following experiments on the human brain, Oliveira et al.[11] verified again that applying SBB evidently decreased autofluorescence, however with an observable loss of intensity of immunocytochemical target labeling. It is worth noticing that increasing exposure time when imaging helps in recognizing fluorescent markers to some degree. For these reasons, we believe applying SBB is worth trying to cut down the influence of lipofuscin-like autofluorescence.

An experiment was designed to verify the effects of SBB, and one hDRG was used (one male donor from the National Human Brain Bank for Development and Function, Chinese Academy of Medical Sciences, and Peking Union Medical College (PUMC) in Beijing, China) to further determine the distribution of ACE2 in sensory neurons. To certify if SBB was appropriate for reducing autofluorescence and the applicability of exposure compensation (EC) for the recognition of fluorescence markers, three experiments were performed. First, two sets of sections were performed with the ordinary procedure of immunofluorescent staining, but without any antibody incubation. One set was treated with SBB (0.1% dissolved in 70% ethanol) for 5 min[10] before being coverslipped with VECTASHIELD Mounting Medium with DAPI. Both sets were scanned at 488 nm and 594 nm stimulation, and exposure time was kept equal for all sections treated with or without SBB. Second, sections were incubated with primary antibody (rabbit anti-ACE2, Abcam, ab15348) and secondary antibody (goat anti-rabbit IgG Alexa Fluor® 488, Abcam, ab150077; Goat anti-Rabbit IgG Alexa Fluor® 594, Abcam, ab150080) with SBB treatment before being sealed. EC was applied to one set of sections as scanned at 488 nm and 594 nm stimulation. Third, sections were incubated with rabbit anti-ACE2 antibody or control rabbit IgG for diaminobenzidine-coupled immunohistochemistry to fundamentally eliminate the interference of autofluorescence. Furthermore, hDRG sections were respectively incubated with ACE2 antibody (rabbit anti-ACE2, Abcam, ab15348) together with CGRP (calcitonin gene-related peptide, goat anti-CGRP, Abcam, ab36001) or NF200 (neurofilament 200, Chicken anti-NF200, Abcam, ab4680). Then, sections were incubated with following corresponding secondary antibodies or Isolectin GS-IB4 Alexa Fluor 594 (Invitrogen, I21413) for 1 h: Donkey anti-rabbit IgG Alexa Fluor 488 (Abcam, ab150073); donkey anti-goat IgG Alexa Fluor 594, Jackson (ImmunoResearch, 705-585-147); and goat anti-chicken IgY Alexa Fluor 594 (Abcam, ab150176).

Fluorescence was detected at both 488 nm and 594 nm, which could be mostly erased by the application of SBB under equal exposure time (Figures 1(a)–(d)). After incubation with antibodies, strong signal was observed at both 488 nm and 594 nm stimulation (Figure 1(e)). Only weak but distinguishable signals were detected after treated by SBB (Figure 1(f)). This phenomenon was consistent with previous reports and compensation of exposure time helped capture sharpened images (Figure 1(g)). The percentage of positive cells was significantly decreased after SBB treatment suggested that SBB prominently reduced the positive signals induced by lipofuscin (Figures 1(h) and (i)). In diaminobenzidine-coupled immunohistochemistry, compared with the negative IgG control, clear ACE2 signals were detected after the application of rabbit anti-ACE2 antibody suggesting the expression of ACE2 in human sensory neurons (Figures 1(j) and (k)). In addition, ACE2 was detected in IB4+ (marker for non-peptidergic nociceptor), CGRP+ (marker for peptidergic nociceptor), and NF200+ (marker for myelinated Aβ-fiber sensors) sensory neurons (Figures 1(l)–(q)), suggesting the potential association between sensory ACE2 and COVID-19related pain.

SBB significantly reduced the massive lipofuscin-like autofluorescence in negative control sections and ACE2 immunofluorescence staining sections. Exposure compensation could offset the ACE2 signal weakened by SBB treatment and would extend the application of SBB. Fluorescence staining and immunohistochemistry showed similar distribution of ACE2 in hDRG. These above results preliminarily verified that SBB reduced autofluorescence in hDRG tissues while causing less impact on staining of target molecule. Lipofuscin deposition occurs not only in the nervous system but also in many other tissues.[12] We believe that attention should be paid to the fluorescence staining experiments of old animal tissues, especially human CNS tissues. Previous studies and our findings suggested that SBB could be an applicable method for controlling autofluorescence in CNS tissues.[13] With the application of SBB, we preliminarily confirmed the distribution of ACE2 in hDRG and demonstrated the co-expression of ACE2 with IB4+, CGRP+, and NF200+ sensory markers. We believe this report contains important implications for further studies of ACE2 distribution and understanding the legacy effects of COVID-19.

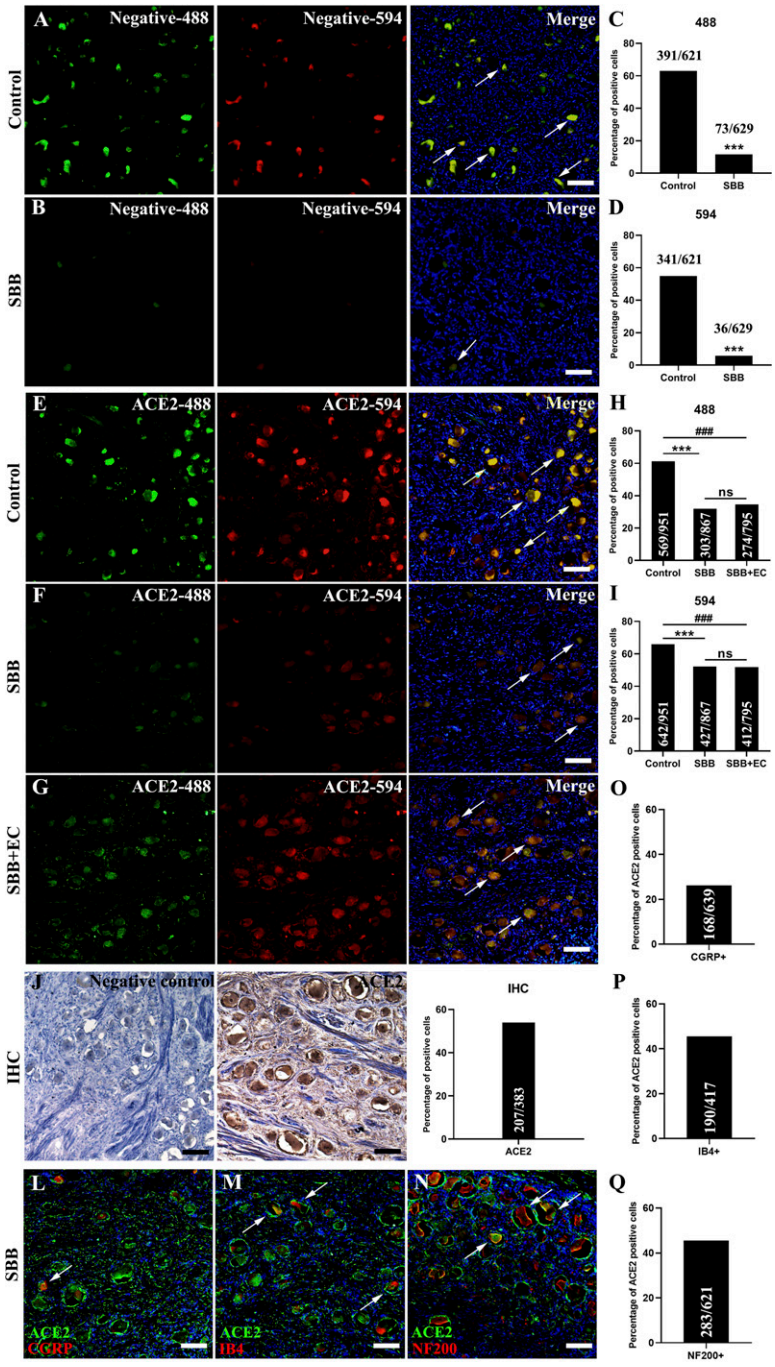

Figure 1. Effect of Sudan Black B treatment on lipofuscin-like autofluorescence of hDRG. (A) Representative images of hDRG section stimulated by 488 nm and 594 nm without any antibody incubation. Scale bar: 100 μm. (B) Representative images of hDRG section stimulated by 488 nm and 594 nm without any antibody incubation and treated by Sudan Black B. Scale bar: 100 μm. (C and D) Treatment of Sudan Black B reduced the lipofuscin-like autofluorescence in hDRG section stimulated by 488 nm and 594 nm without any antibody incubation. ***$p < 0.001$, chi-square test, SBB versus Control. (E) Representative images of hDRG section stimulated by 488 nm and 594 nm incubated by rabbit anti-ACE2 antibody. Scale bar: 100 μm. (F) Representative images of hDRG section captured under equal exposure time incubated by rabbit anti-ACE2 antibody and treated by Sudan Black B. Scale bar: 100 μm. (G) Representative images of hDRG section captured with exposure compensation (EC) incubated by rabbit anti-ACE2 antibody and treated by Sudan Black B. Scale bar: 100 μm. (H–I) Treatment of Sudan Black B reduced the lipofuscin-like autofluorescence as immunofluorescence applied. ***$p < 0.001$, chi-square test, SBB versus Control. ***$p < 0.001$, chi-square test, SBB+EC versus Control. ns, no significance, chi-square test, SBB versus SBB+EC. (J) Immunohistochemical staining showed the existence of ACE2 in hDRG. Scale bar: 100 μm. (K) Summation of ACE2+ cells detected by immunohistochemical staining in hDRG. (L–N) Representative microscopic images of immunostaining for ACE2 co-expression with CGRP, IB4, and NF200 in hDRG treated with SBB. Positive cells shown by arrows. Scale bar: 100 μm. (O–Q) Percentages of ACE2+ cells in hDRG with sensory neuronal markers (CGRP, IB4, and NF200). Scale bar: 100 μm.

Author Contributions

SS and NY designed and performed the experiments and then acquired the data. SS and HZ contributed to data analysis and interpretation. All authors read and approved the final manuscript.

Declaration of conflicting interests

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Ethical approval

The studies involving human participants were reviewed and approved by the Institutional Review Board of the Institute of Basic Medical Sciences of the Chinese Academy of Medical Sciences, PUMC, Beijing, China (approval number: 009-2014).

Informed consent

The patients/participants provided their written informed consent to participate in this study.

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