Substance P reversibly compromises the integrity and function of blood-brain barrier

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Abstract

Background: Substance P (SP) plays a role in vasodilatation and tissue integrity through its receptor, neurokinin 1 (NK1R). However, its specific effect on blood-brain barrier (BBB) remains unknown.

Methods: The impact of SP on the integrity/function of human BBB model *in vitro*, composed of brain microvascular endothelial cells (BMECs), astrocytes and pericytes, was assessed by measurements of transendothelial electrical resistance and paracellular flux of sodium fluorescein (NaF), respectively in the absence/presence of specific inhibitors targeting NK1R (CP96345), Rho-associated protein kinase (ROCK; Y27632) and nitric oxide synthase (NOS; N(G)-nitro-L-arginine methyl ester). Sodium nitroprusside (SNP), a NO donor, was employed as a positive control. The levels of tight junction proteins, zonula occludens-1, occludin and claudin-5 alongside RhoA/ROCK/myosin regulatory light chain-2 (MLC2) and extracellular signal-regulated protein kinase (Erk1/2) proteins were detected by western analyses. Subcellular localisations of F-actin and tight junction proteins were visualized by immunocytochemistry. Flow cytometry was used to detect transient calcium release.

Results: Exposure to SP increased RhoA, ROCK2 and phosphorylated serine-19 MLC2 protein levels and Erk1/2 phosphorylation in BMECs which were abolished by CP96345. These increases were independent of the changes in intracellular calcium availability. SP perturbed BBB in a time-dependent fashion through induction of stress fibres. Changes in tight junction protein dissolution or relocalisation were not involved in SP-mediated BBB breakdown. Inhibition of NOS, ROCK and NK1R mitigated the effect of SP on BBB characteristics and stress fibre formation.

Conclusion: SP promoted a reversible decline in BBB integrity independent of tight junction proteins expression or localisation.

Keywords: substance P, brain microvascular endothelial cells, blood-brain barrier, RhoA, tight junctions; neurokinin 1 receptor; stress fibres

Introduction

Substance P (SP) is a neurotransmitter reported to mediate nociception through its receptor, neurokinin-1 (NK1R) [1]. The distribution and function of SP has been studied in various systems including cardiovascular [2], gastrointestinal [3] and immune [4] systems. In central nervous system, most SP-related studies to date have focused mainly on behavioural regulation [5].

As evidenced by widespread distribution of NK1R in various endothelial cell (EC) lines, SP plays a pivotal role in endothelial (cell) function [6]. For instance, SP regulates the growth and proliferation of pulmonary artery ECs [7] and negates the suppressive effects of pro-inflammatory cytokine, tumour necrosis factor-alpha (TNF-α) on nitric oxide (NO) production in human umbilical vein ECs [8]. In addition, SP has been shown to trigger rapid expression of endothelial leucocyte adhesion molecule-1 in neonatal human foreskin-derived adjacent post-capillary venular ECs [9] and promote the leakage of plasma through ECs in rat tracheal mucosa [6].

Studies focusing on the role of SP in neurological disease has shown marked progression of brain oedema and dysfunction after traumatic brain injury [10]. Similarly, intravenous injection of SP to rats increased plasma extravasation in the dura matter [11]. Treatments with NK1R antagonists, N-acetyl-l-tryptophan or Emend, on the other hand were shown to reduce permeability in a rodent model of middle cerebral artery occlusion and decrease blood-brain barrier (BBB) permeability [12].

Scrutiny of the specific impact of SP on BBB or brain microvascular ECs (BMECs) revealed the role of SP in HIV-1 Gp120-mediated infection of BBB *in vitro* [13] and in human NK1R-dependent BMEC barrier dysfunction [14]. Neutralisation of interferon-γ- and TNF-α-

mediated rodent BBB hyperpermeability by Spantide, a SP antagonist, support these findings [15].

In addition to endothelial regulation, the G protein coupled receptor, NK1R, also modulates the anti-inflammatory, anti-apoptotic, vasodilatory [16], proliferative and chemotactic effects [17] of SP through activation of an array of signalling pathways such as Ras homolog family member A (RhoA)/Rho-associated protein kinase (ROCK)/myosin light chain (MLC) [18], extracellular signal-regulated protein kinase (Erk1/2) [19] and NO synthase/NO [20].

In light of the above, this study investigated the specific effect of SP on BBB integrity and function *in vitro* with reference to putative involvements of RhoA/ROCK/MLC, NOS, Erk1/2 and calcium signalling pathways and associated morphological phenomena. The triple cell culture model of human BBB has been used in the current study. This is a well-established model that closely mimics the characteristics of human cerebral barrier in both physiological and pathological settings [21, 22].

Methods

Cell culture and materials

Primary human BMECs (HBMEC), human astrocytes (HA) and human pericytes (HP) were purchased from Neuromics, USA. Cells were seeded on T75 cm^2 flask (Corning), in respective complete medium supplemented with growth factor and 1% Penicillin/Streptomycin Solution (Sciencell, USA), and incubated at 37ºC with 5% CO_2/95% air and saturated humidity.

A triple culture model of human BBB, composed of HBMEC, HA and HP, was established as before [23]. Briefly, HA were seeded on the abluminal side of 12 well transwell (12mm diameter inserts, 0.4 µm pore size, Corning) inserts and allowed to attach membrane for 4 h before inverting them the right way. When the astrocytes reached ~80% confluence, the HBMEC were seeded on luminal side of the inserts and both layers were cultured to full

confluence. The inserts were then transferred to fresh 12-well plates containing confluent pericytes to establish triple cell culture model of human BBB. In this study, cells were treated with SP (1µM, Sigma, USA) or a NO donor, sodium nitroprusside (SNP, 1µM, Merck millipore, USA), in the absence or presence of inhibitors for NOS (N(G)-nitro-L-arginine methyl ester or L-NAME, 10 µM, Abcam, UK) or ROCK (Y-27632, 2.5 µM, Abcam, UK) or an NK1R antagonist (CP96345, 10 µM, R&D Systems, USA).

Evaluation of BBB integrity and function

The integrity of the BBB was assessed by measurement of transendothelial electrical resistance (TEER) using an EVOM resistance meter (World Precision Instruments, Hertfordshire, UK) as before [23]. BBB permeability was examined as before [24] where sodium fluorescein (NaF) was added to apical chamber and incubated for 1 h at 37℃ before collection of buffer from both upper and lower chamber. Then 500 µL samples were read by fluorometer (excitation/emission: 440/525 nm) in 96 well plate (100 µl/well). NaF flux was calculated with following formula, NaF cleared volume (µL) = abluminal reading x 500/luminal reading.

Western blot

After exposure to experimental conditions, cells were trypsinized and washed with phosphate-buffered saline (PBS), before lysis in radioimmunoprecipitation assay buffer containing phenylmethylsulfonyl fluoride (both from Cell Signalling Technology, USA), PhosSTOP, complete EDTA-free protease inhibitor (both from Roche Diagnostics GmbH, Germany). The cell lysates were stored in -80℃. Protein concentrations were determined by bicinchoninic acid assay (Thermo scientific, USA) and read at 562nm on a microplate reader 680 (Bio-Rad, USA). The standard curve (BSA standards in range 0-2.0 mg/ml) was plotted, and total concentration was calculated accordingly.

At least 50-80 μg of total protein samples was loaded and separated on a 6-12% sodium dodecyl sulphate-polyacrylamide gel. After proteins were transferred, membranes were blocked and incubated with primary antibodies raised against GAPDH (Sigma Aldrich, UK, HPA040067 and G8795, both 1:1000), RhoA, ROCK2 (Santa Cruz, USA, sc-418 and sc-398519, both 1:150), phospho-Threonine18Serine19 MLC2 (PThr18/Ser19) and phospho-Serine19 MLC2 (PSer19) (Cell Signalling Technology, USA, #3674 and #3671, both 1:200), MLC2 (Merck Millipore, USA, MABT180, 1:2000), ZO-1, occludin, claudin-5 (Invitrogen, USA, # 33-9100, # 71-1500 and # 35-2500, 1:250,1:250 and 1:1000), phospho-Erk1/2, Erk1/2 (Cell Signalling Technology, USA, #9101 and #9102, both 1:000) and NK1R (Abcam, UK, ab183713, 1:10000) overnight at 4°C. After washing, the membranes were incubated with secondary antibodies (anti-mouse IgG and anti-rabbit IgG, Licor Biosciences, UK, 926-68072 and 926-32213, both 1:15000) for 1h at room temperature then washed and developed using Li-Cor Odyssey infrared imaging system (Li-Cor Biosciences, UK). Relative abundance of proteins was analysed by Image Studio Lite (Li-Cor Biosciences, UK).

Immunocytochemistry

HBMECs were grown on coverslip and subjected to respective experimental conditions. The cells were then washed with PBS and successively fixed and permeabilised in 4% paraformaldehyde solution in PBS (Thermo Fisher, USA) and 0.1% Triton X-100. To visualise stress fibres, the cells were incubated with phalloidin-iFluor 647 Reagent (Abcam, UK, ab176759, 1:1000). Tight junction proteins, ZO-1, occludin and claudin-5 were stained successively with relevant primary (Invitrogen, USA, # 33-9100, # 71-1500 and # 35-2500, all 1:100) and secondary antibodies (anti-mouse IgG and anti-rabbit IgG, Abcam, UK, ab150113 and ab150077, both 1:1000). Coverslips were then counterstained by mounting medium VECTASHIELD Antifade (Vector Laboratories, USA) containing 4,6-diamidino-2-phenylindole (DAPI) to mark nuclei.

Rho activity assay

Rho activation was examined by Rho-pull down kit (Merck Millipore, USA). In brief, glutathione-tagged agarose beads fused with Rhotekin Rho Binding Domain were incubated with at least 150 μg cell lysates. Western blot was then performed to detect active RhoA, RhoB, and RhoC [25].

Calcium release measurement

Intracellular calcium release was detected by flow cytometry with Spectral flow cytometer ID7000 (Sony Biotechnology, USA). Baseline was determined by monitoring calcium level from samples of fluorescent calcium indicator-labelled (Fluo-4 AM, Invitrogen, USA, F14201,1μM) cells for 60 s. Then SP was added to cells without unplugging the tube and signal was acquired for another 6 min. Flow cytometry standard files were analysed using FACS Diva software (BD Biosciences, USA).

Statistical analysis

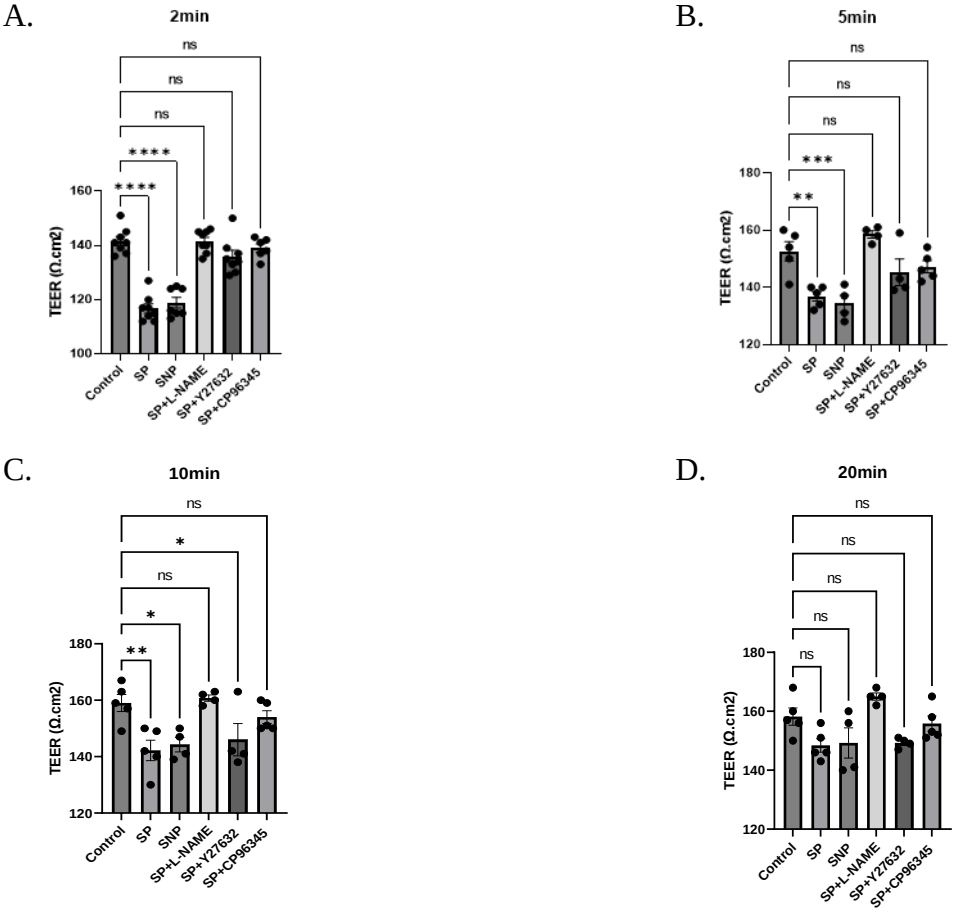
Data were analysed using the GraphPad Prism 9.4.1 software. One-way ANOVA was used for analyses. The post hoc test Dunnett were carried out. P-value <0.05 was considered statistically significant. The data were recorded as the mean±s.e.m. from at least 3 independent experiments.

Results

Effects of SP and on BBB integrity and function

SP significantly perturbed BBB integrity as ascertained by significant decreases in TEER readings after 2, 5 and 10 min of exposure (F=38.99, df=39, P<0.0001 at 2 min, F=10.13, df=21, P=0.002 at 5 min, F=5.743, df=21, P=0.0048 at 10 min) which were negated by inhibition of NOS and NK1R at all timepoints. In contrast, inhibition of ROCK2 appeared to neutralise the BBB-disruptive effects of SP at the shorter time point studied, 2 and 5 min. No

changes in TEER were observed in studies where BBB was exposed to SP for 20 min or more (F=5.141, df=21, P=0.0681 at 20 min). Exposure to SNP, produced similar responses to those evoked by SP, ascribing a role to NO (F=38.99, df=39, P <0.0001 at 2 min, F=10.13, df=21, P=0.0008 at 5 min, F=5.743, df=21, P=0.0212 at 10 min, F=5.141, df=21, P=0.1378 at 20 min) (Fig. 1A-D). Post hoc analyses showed a significant decrease in SP and SNP groups compared to control groups. SP also impaired BBB function in a time-dependent fashion as evidenced by increases in NaF flux solely after 2 min of exposure (F=8.754, df=18, P=0.0284 at 2 min, F=2.703, df=18, P=0.1594 at 20 min). Post hoc analyses showed a significant increase in SP groups compared to control groups. Co-treatments with L-NAME, Y27632 and CP96345 abolished the effect of SP on barrier function. The effects of SNP on BBB were similar to that of SP at 2 and 20 min of exposure (Fig. 1E-F).



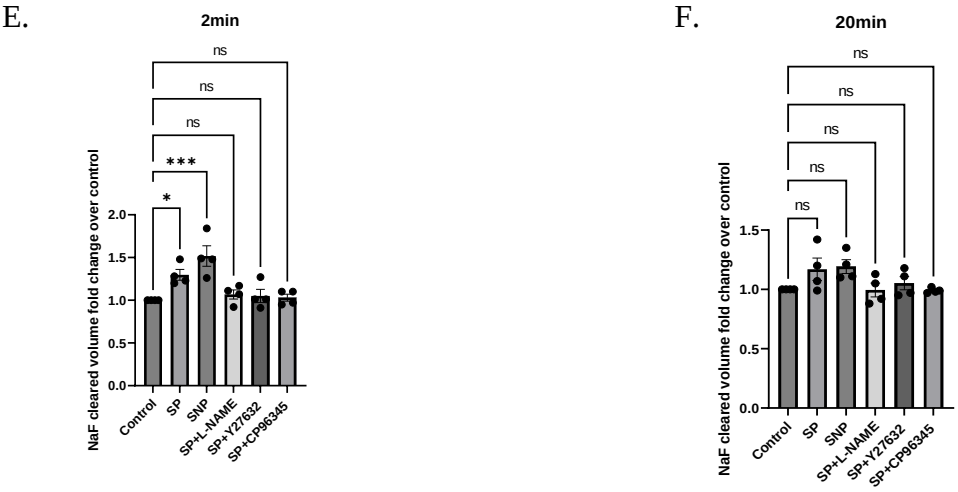
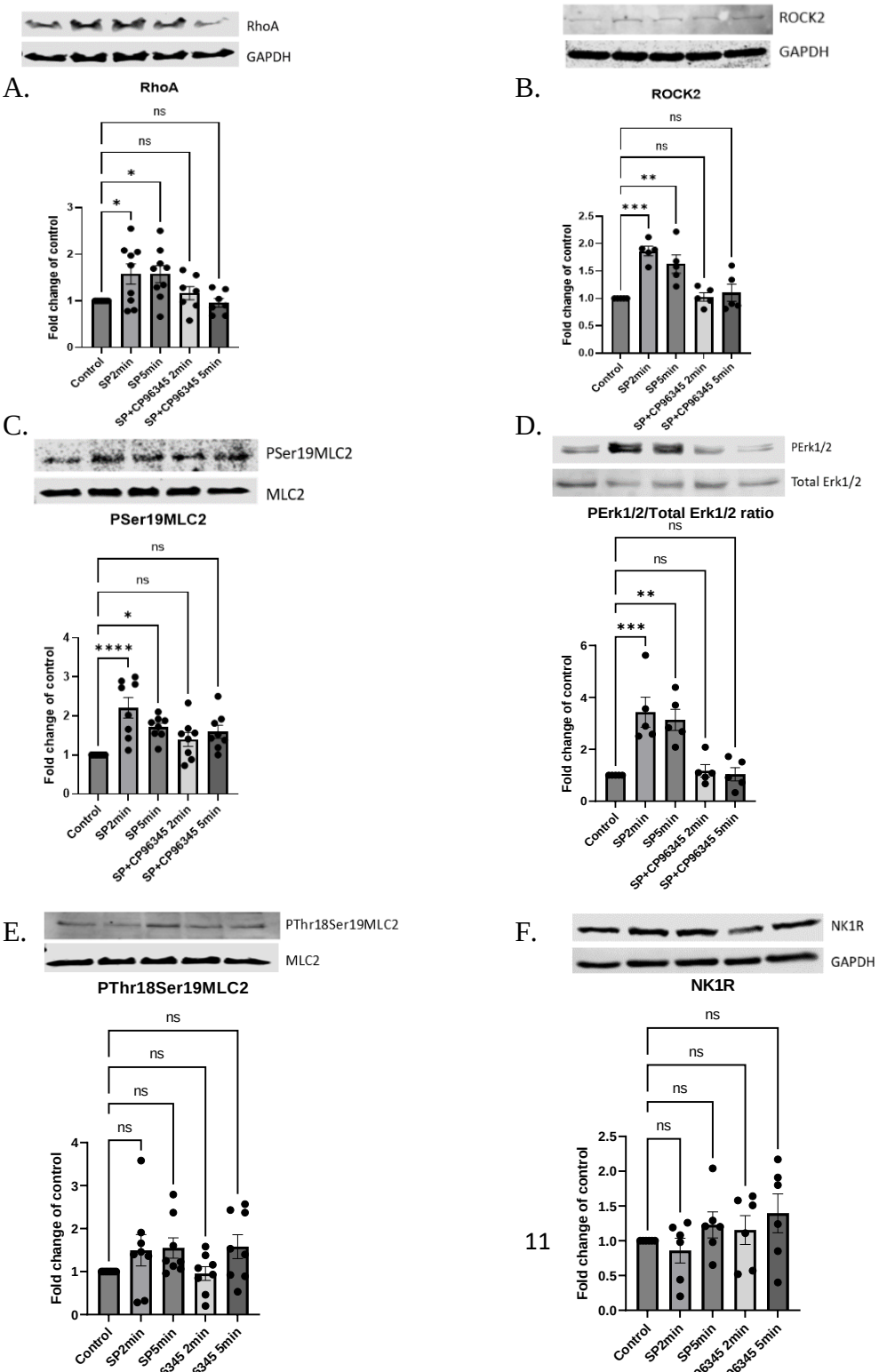


Fig. 1. Substance P (SP) impairs the characteristics of blood-brain barrier (BBB) *in vitro*. The integrity of the BBB was compromised after 2 min (**A**), 5 min (**B**), 10 min (**C**) but not 20 min (**D**) of exposure to SP or nitric oxide donor, sodium nitroprusside (SNP). Both SP and SNP also impaired the function of BBB as evidenced by time-dependent increases in paracellular flux of sodium fluorescein (NaF) after 2 min (E) but not 20 min (F) incubation with either compound. Co-treatment of BBB with specific inhibitors for nitric oxide synthase (L-NAME) and neurokinin 1 receptor (CP96345) blocked SP-evoked changes in TEER and NaF flux at all time points other than 20 min. Treatment with a Rho kinase inhibitor (Y27632) mitigated the effect of SP selectively after 2 and 5min of exposure. Data are shown as mean±s.e.m. from at least three different experiments. *p<0.05, **p<0.01, ***p<0.001.

Effects of SP on expression of RhoA/ROCK/MLC2 pathway, Erk1/2, NK1R and tight junction proteins

Treatments with SP for 2 min or 5 min produced significant increases in RhoA (F=3.98, df=36, P=0.0306 at 2 min, P=0.031 at 5 min), ROCK2 (F=11.91, df=20, P=0.0001 at 2 min, P=0.0035 at 5 min), phosphorylated Ser19 MLC2 (PSer19MLC2) (F=6.947, df=35, P<0.0001 at 2 min, P=0.0174 at 5 min) and phosphorylated Erk1/2 (PErk1/2) (F=11.95, df=20, P=0.0004 at 2 min, P=0.0013 at 5 min) (Fig. 2A-D), but not PThr18Ser19MLC2 (F=1.647, df=35, P=0.4129 at 2 min, P=0.3267 at 5 min) and NK1R (F=1.139, df=25, P=0.959 at 2 min, P=0.8201 at 5 min)

protein expressions (Fig. 2E-F). Post hoc analyses showed upregulation of RhoA, ROCK2, PErk1/2 and PSer19MLC2 protein expression in SP treated groups compared to untreated groups. Co-treatment of cells with CP96345 neutralised all these increases. In contrast, exposure to SP for 2 min or 5 min did not affect the expression of tight junction proteins, ZO-1 (F=0.6774, df=20, P=0.9999 at 2 min, P=0.7298 at 5 min) , occludin (F=1.056, df=30, P=0.4201 at 2 min, P=0.6197 at 5 min), or claudin-5 (F=0.8428, df=25, P=0.9999 at 2 min, P=0.7169 at 5 min) in HBMECs (Fig. 2G-I).



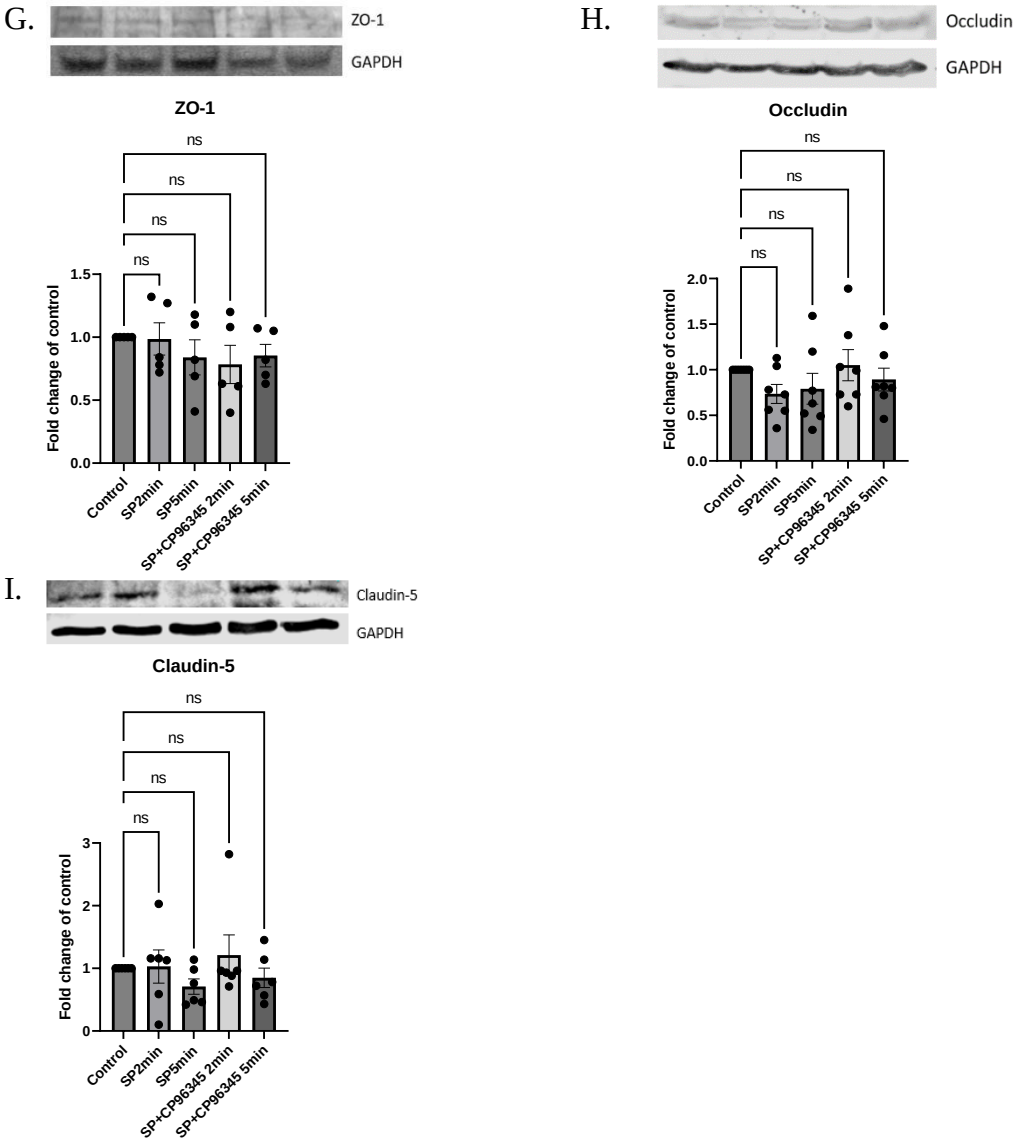


Fig. 2. Substance P (SP) differentially regulates the expression of proteins associated with blood-brain barrier integrity. Treatment of human brain microvascular endothelial cells with SP for 2 min and 5 min significantly enhanced the protein expressions of RhoA (**A**), ROCK2 (**B**), PSer19MLC2 (**C**), PErk1/2 (**D**), but not those of PThr18Ser19MLC2 (**E**), neurokinin 1 receptor (**F**), zonula occludens-1 (**G**), occludin (**H**) and claudin-5 (**I**). Co-treatment of cells with neurokinin 1 receptor antagonist, CP96345 negated the effects of SP on all proteins. Data are expressed as mean±s.e.m. from at least three different experiments. *p<0.05, **p<0.01, ***p<0.001.

Impact of SP on cytoskeleton and subcellular localisation of tight junction proteins

Exposure of HBMECs to SP led to translocation of normally plasma membrane-bound actin filaments to cytoplasm and generation of stress fibres traversing the cells. Inhibition of NK1R prevented SP-mediated stress fibre formation and restored plasma membrane staining of actin fibres (Fig. 3A). Amongst the three TJ proteins studied, only ZO-1 has been detected on the plasma membrane. Co-treatments with CP96345 did not have any impact on subcellular localisation of ZO-1 (Fig. 3B). Neither occludin nor claudin-5 could be detected on plasma membrane in control HBMECs, rather they were detected in cytoplasm. Unexpectedly, both proteins showed a prominent nuclear staining solely in SP-treated cells. Inhibition of NK1R in SP-treated cells with CP96345 successfully returned both proteins' staining to cytoplasm (Fig. 3C-D).

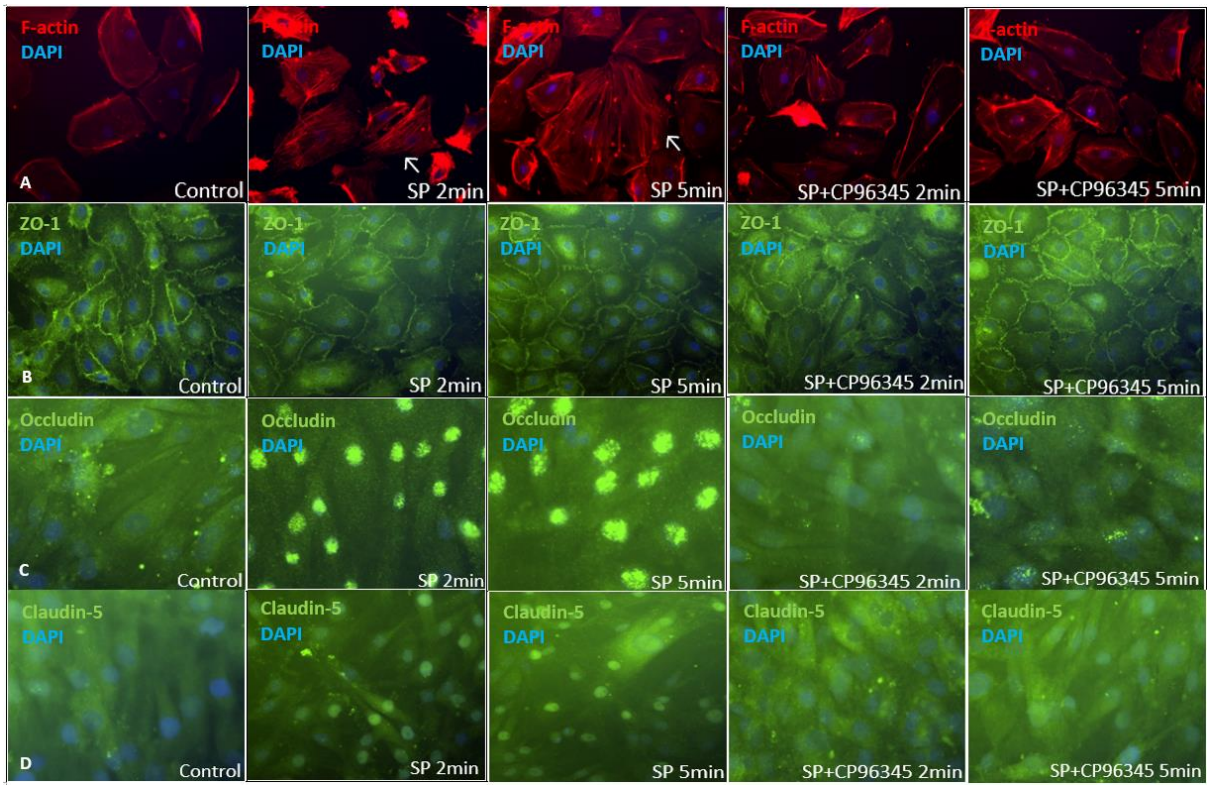


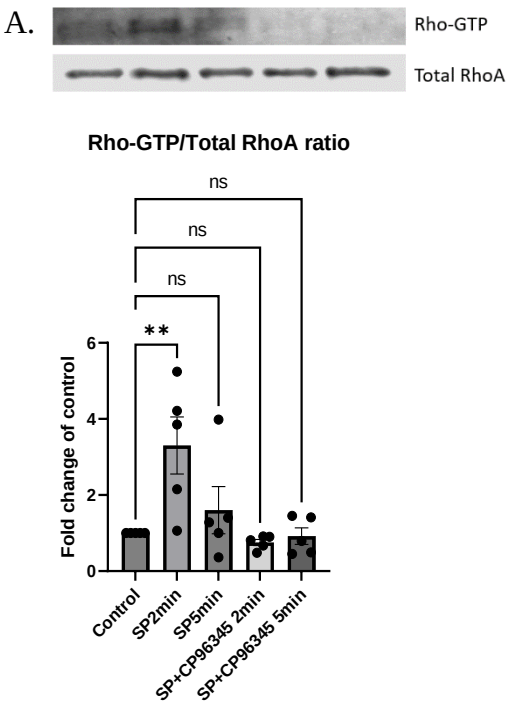
Fig. 3. Substance P (SP) influences the subcellular localisation of microfilaments and tight junction proteins (red for F-actin, green for ZO-1/Occludin/Claudin-5, blue for DAPI). Under normal conditions, human brain microvascular endothelial cells (HBMECs) exhibit membrane-

bound actin staining. Exposure to SP for 2 min or 5 min led to development of stress fibres in these cells that were prevented by an antagonist of neurokinin 1 receptor, CP96345 (**A**). No difference in plasma membrane localisation of zonula occludens-1 (ZO-1) was observed in HBMECs subjected to SP alone or with CP96345 (**B**). Instead of plasma membrane, both occludin and claudin-5 appeared to localise cytosol. Treatments with SP translocated these proteins to nuclei. Co-treatment with CP96345 restored the cytosolic localisation of both proteins (**C, D**).

Impact of SP on endothelial cell Rho activation and calcium release

Treatment of HBMEC with SP for 2 min elicited upregulation of Rho-GTP or Rho-GTP/total RhoA levels (F =5.499, df =20, P=0.0058). Post hoc analyses showed a significant increase in SP 2 min treatment groups compared to control groups. Targetting NK1R in the same experiments did not significantly alter any of the above elements (Fig. 4A).

To capture possible changes in intracellular calcium levels in HBMEC subjected to SP, first calcium signal was collected for 60 s to set up a baseline which was followed by the addition of SP and monitoring of potential fluctuations in calcium levels for 6 min. No difference in calcium levels was observed before or after addition of SP (Fig. 4B).



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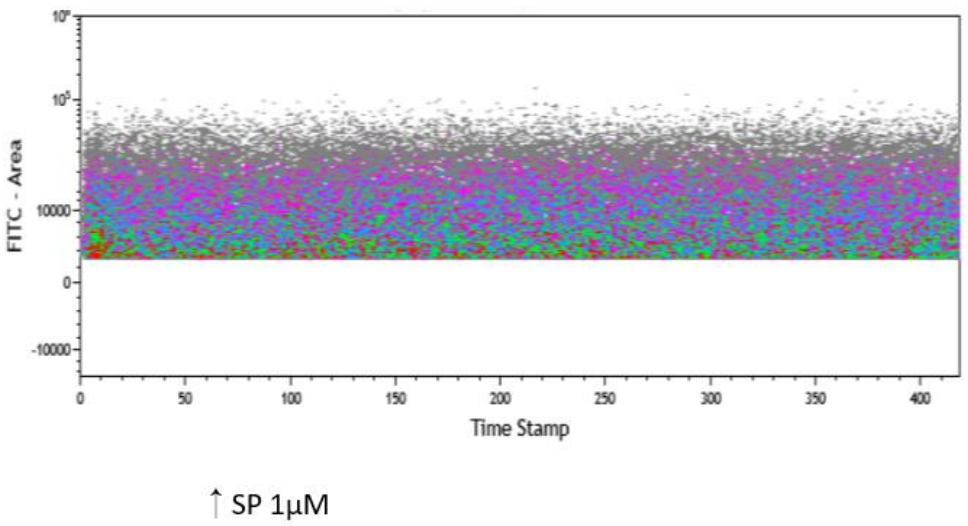


Fig. 4. Effect of substance P (SP) on activated Rho (Rho-GTP) and intracellular calcium levels. Human brain microvascular endothelial cells produced higher levels of Rho-GTP in response to treatments with SP for 2 min. Co-treatments with CP96345, a neurokinin 1 receptor antagonist, did not influence either parameter (**A**). Continuous exposure to SP for 6 min did not induce mobilisation of intracellular calcium (**B**). Data are expressed as mean±s.e.m. from three different experiments. ***p*<0.01.

Discussion

The main findings of this study were that SP, a neurotransmitter and a neuromodulator, exerts a reversible disruptive effect on the integrity and function of an *in vitro* model of human BBB through a mechanism involving changes in protein expression of endothelial NK1R, NOS, RhoA/ROCK2 and Erk1/2. Previous data concerning the role of SP in cerebral circulation come mostly from *in vivo* studies and are rather controversial in that intravenous injection of SP has been shown to increase or maintain cerebrovascular permeability as ascertained by detection or no detection of permeability markers, Evans Blue and sulphorhodamine B, in the brain parenchyma [26, 27]. In the present study, treatments with exogenous SP consistently but transiently compromised BBB integrity and function, starting 2 min after exposure and completely disappearing after 20 min. These were similar to the responses generated by a NO

donor i.e. SNP [28]. SP-mediated BBB hyperpermeability was previously reported in an *in vitro* model cerebral barrier established with human induced pluripotent stem cell-derived BMECs [14, 29]. Normalisation of NaF flux following 5 min of exposure to SP in the present study, despite continuing decreases in TEER, imply the existence of very small inter-endothelial cellular openings after this time. For TEER measurements, a small alternating current is passed from one electrode to another to detect how much of electrical signal is blocked by the cellular layer thereby quantifying the barrier integrity. Though they may be small enough to prevent the passage of NaF across the barrier, these intercellular openings continue to negatively affect TEER until they are closed/repaired in full [30]. Similar to this study, SP has been shown to evoke much of the cerebral oedema *in vivo* at 2 min and lost 50% of its permeability-increasing activity at 2.7 ± 0.1 min [31].

To unravel the potential mechanisms behind the SP-mediated BBB damage, the involvement of key mechanisms proposed to be involved in pathophysiological functions of SP, namely NOS, NK1R and RhoA/ROCK pathway were examined using the *in vitro* BBB model and HBMECs, the main cellular component of the BBB. As a non-selective NOS inhibitor, L-NAME has been implicated in various SP-related biological activities such as relaxation of precontracted airway smooth muscle [32], we tested its efficacy in our BBB model. Similar to previous studies reporting an effective suppression of BBB *in vitro* [33], L-NAME also attenuated SP-induced BBB leakage in our model. Taken together these findings bestow a crucial role on NO in the modulation of SP-related cerebral microvascular dysfunction.

Guanosine triphosphate-binding protein, RhoA, exert crucial roles in many cellular processes, including the regulation of endothelial barrier integrity and function [34]. Once activated, RhoA binds to its downstream effector ROCK and as a consequence induce myosin-regulatory light chain-2 phosphorylation (p-MLC2) and actin stress fibre formation in a sequential manner. Because p-MLC2 destabilizes cell junctions to increase paracellular flux, the

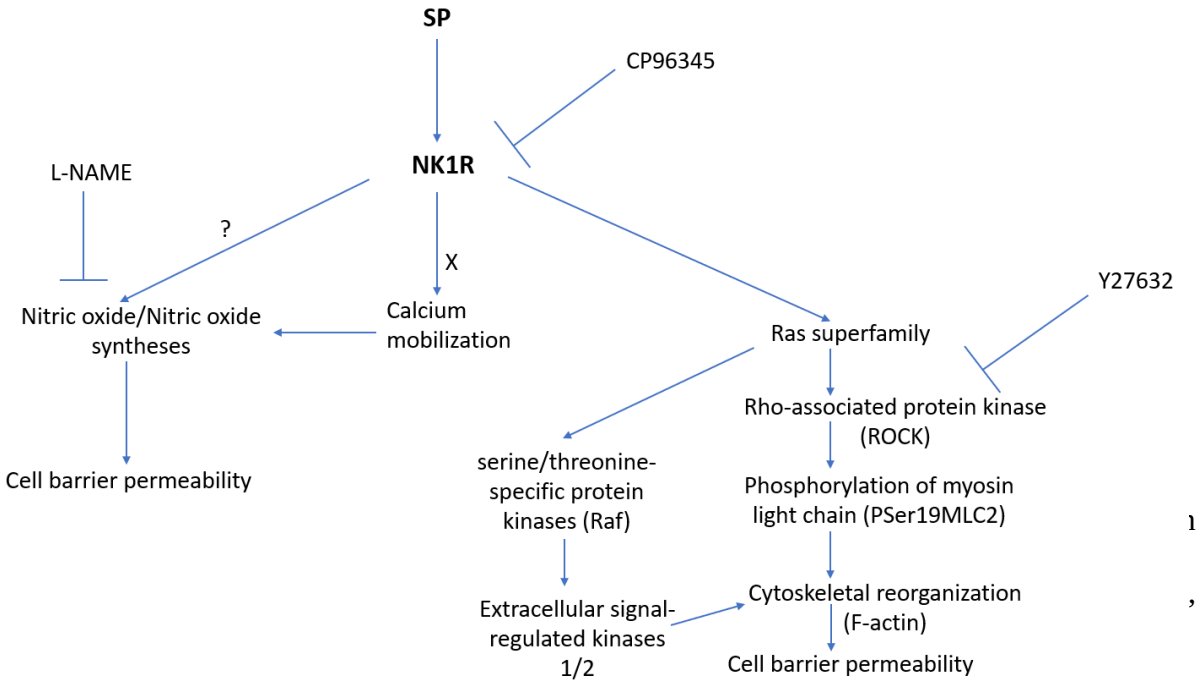
inhibition of Rho/ROCK pathway may improve microvascular endothelial function and suppress barrier permeability [25]. Suppression of SP-related barrier openings at 2 min by Y27632, a ROCK inhibitor indicates the involvement of RhoA/ROCK pathway in early phases of this process. Having established the involvement of RhoA/ROCK/pMLC2 pathway in SP-mediated barrier damage, we then looked at the expression of respective proteins in control HBMECs and those treated with SP. These revealed significant increases in RhoA, ROCK and PSer19MLC2 levels in HBMECs treated with SP for 2 min or 5 min, similar to previous studies [35]. In addition to a previous report showing the SP-mediated RhoA activation in human colonic epithelial cells [36], increases in SP-triggered Rho-GTPase activity in HBMEC further strengthens the role of RhoA/ROCK pathway in SP-related biological effects. Even so, like arachidonic acid, SP may modulate ROCK2 expression and activity independent of RhoA [37, 38]. Indeed, higher protein expression - and potential activity of ROCK2 - along with the increase observed in PSer19MLC2 may be sufficient to impair BBB integrity and function [39].

As attested in lymphatic muscle cells and BMECs, SP-mediated overactivation of Erk1/2 may also contribute to MLC2 phosphorylation and exacerbate BBB damage [40]. Erk1/2 pathway modulates phosphorylation of MLC2 by decreasing the activity of MLC Phosphatase (MLCP) [41]. Inability of SP to enhance intracellular calcium levels and activate RhoA in HBMECs may explain the lack of Thr18Ser19MLC2 phosphorylation in our study. Indeed, phosphorylation of MLC2 requires either activation of MLC kinase (MLCK) by calcium or inhibition of MLCP by ROCK [42]. Similar to MLCK, ROCK can also directly phosphorylate MLC2 at Ser19 [43]. Phosphorylation of Thr18 follows the phosphorylation of Ser19 and requires substantially higher concentrations of MLCK [44].

Scrutiny of the impact of SP on tight junction proteins, known to prevent paracellular leakage, indicate that SP does not affect either protein expression or subcellular localisation of ZO-1,

claudin-5 or occludin. Similar observations regarding the effects of SP on barrier integrity and stress fibres formation in the absence of tight junction protein modulation were previously reported by another group [14]. An increase in BBB penetration in the absence of changes in tight junction proteins was also reported in an *in vivo* study employing leptin-deficient mice [45]. It is probable that intercellular gap formation, stemming from tight junction protein breakdown, may require MLC2 diphosphorylation [46]. The lack of fenestrae and extremely low pinocytotic activity in BMECs inhibit transcellular passage of molecules across the BBB. Even so, mechanisms that do not require the breakdown of tight junctional unity such as activation of ion/water channels and endothelial glycocalyx destruction may be involved in SP-mediated increases in NaF flux as observed in an animal model of human transient ischaemic stroke [47]. Caveolae-based transcytosis may also play a role in this process. Caveolae are known to be particularly abundant in endothelial cells where they play a key role in the regulation of endothelial vesicular trafficking and signal transduction. Caveolae also play a role in mechanosensitive cellular pathways, including RhoA-driven actomyosin contractility and are frequently associated with stress fibres [48]. In the present study, SP generated thick actin stress fibres traversing the cells. By creating a tensile centripetal force, stress fibres pull tight junction proteins inward and thus widen inter-endothelial cell gaps and compromise junctional integrity [34]. Somewhat unexpected nuclear and cytoplasmic localisation of claudin-5 and occludin in HBMECs in the absence or presence of SP may also contribute to the BBB openings. Non-plasma membrane localisation of occludin has previously been noted in HBMECs [49]. NK1R are localised to caveolae and the fact that CP96345 reduces SP-evoked stress fibre formation and NaF flux well before the TEER values are normalised may suggest the involvement of caveolae-based transcytosis in BBB hyperpermeability [50]. However, it is noteworthy that despite shedding some light on the increased BBB permeability observed in this study, these findings cannot satisfactorily explain the absence of occludin and

claudin-5 in BMEC plasma membrane. The proposed signalling pathways activated by NK1R on HBMEC in our findings are summarized in figure 5.



Conclusion

In this study, we found that SP affects the function of BMECs and an *in vitro* model of BBB through activation of NK1R. Activation of RhoA/ROCK/MLC2 signalling pathway along with NO and Erk1/2 appeared to account for at least some of the change observed in BBB integrity and function. Detailed future studies focusing on each of the abovementioned individual mechanisms are required to delineate the precise role of these mechanisms in SP-mediated breakdown of the BBB and recovery from it.

Declaration of competing interest

The authors have no competing interests or personal relationships that could have appeared to influence the work to declare.

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