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Acute downregulation of novel hypothalamic protein sushi repeat-containing protein X-Linked 2 (SRPX2) after experimental traumatic brain injury

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Running Title: SRPX2 expression after traumatic brain injury

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Abstract

Traumatic brain injury (TBI) causes damage to the hypothalamo-hypophyseal axis, leading to endocrine dysregulation in up to 40% of TBI patients. Hence, there is an urgent need to identify non-invasive biomarkers for TBI-associated hypothalamo-hypophyseal pathology. Sushi repeat-containing protein X-linked 2 (SRPX2) is a novel hypothalamic protein expressed in both rat and human brain. Our objective was to investigate the effect of acquired brain injury on plasma SRPX2 protein levels and SRPX2 expression in the brain. We induced severe lateral fluid-percussion injury in adult male rats and investigated changes in SRPX2 expression at 2 h, 6 h, 24 h, 48 h, 72 h, 5 d, 7 d, 14 d, 1 month, and 3 months post-injury. The plasma SRPX2 level was assessed by Western blot analysis. Hypothalamic SRPX2-immunoreactive neuronal numbers were estimated from immunostained preparations. At 2 h post-TBI, plasma SRPX2 levels were markedly decreased compared with the naïve group (AUC=1.00, $p<0.05$). Severe TBI caused a reduction in the number of hypothalamic SRPX2-immunoreactive neurons bilaterally at 2 h post-TBI as compared to naïve group (5032 ± 527 vs 9440 ± 351, $p<0.05$). At 1 month after severe TBI, however, the brain and plasma SRPX2 levels were comparable between the TBI and naïve groups ($p>0.05$). Unsupervised hierarchical clustering using SRPX2 expression differentiated animals into injured and uninjured clusters. Our findings indicate that TBI leads to an acute reduction in SRPX2 protein expression and reduced plasma SRPX2 level may serve as a candidate biomarker of hypothalamic injury.

Key words: Hypothalamus, lateral fluid-percussion injury, sushi repeat-containing protein X-Linked 2, traumatic brain injury

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Introduction

Each year an estimated 69 million people experience traumatic brain injury (TBI) worldwide.[1] TBI is caused by a mechanical insult to the brain due to external forces, including blow to the head, concussive forces, blasts, and bullet injuries, and patients experience chronic neurobiological, psychological and social abnormalities after brain injury.[2] Hypothalamo-pituitary axis dysfunction is a common sequel of TBI in humans.[3–7] Hypopituitarism is prevalent in 11-69 % of adults after TBI,[8–10] and abnormalities in the hypothalamo-pituitary-adrenal (HPA) axis after TBI are well described.[11] However, TBI-induced damage to the hypothalamo-pituitary axis remains difficult to identify due to lack of non-invasive biomarkers of hypothalamic injury.

Like in primates, the hypothalomo-pituitary complex is located at the bottom of the skull in rodents, and is thus, subject to physical force-induced injury. Several studies have demonstrated that the lateral fluid-percussion injury (FPI) model of TBI leads to hypothalamic damage in rodents. For example, lateral FPI in rats causes bilateral acute mild hemorrhage ventral to the paraventricular nucleus in hypothalamus at 1 h post-FPI.[12] Additionally, disruption of sleep-wake behavior and a reduction in orexin-A positive neurons in the lateral hypothalamus are observed in rats 1 month after lateral FPI.[13] These findings suggest that lateral FPI provides a relevant platform for the discovery of biomarkers indicative of hypothalamo-pituitary damage.

Sushi repeat-containing protein X-linked 2 (SRPX2) is a novel protein associated with language development, synaptic plasticity, tissue remodeling and angiogenesis.[14–17] It was first identified in t(17;19)-positive leukemia cells as a downstream molecule of the E2A-HLF fusion gene.[18] Subsequent findings indicated that mutations (Y722S or N327S) of the *SRPX2* gene are associated with speech dyspraxia, mental retardation, bilateral perisylvian polymicrogyria and Rolandic epilepsy.[17] Furthermore, it was shown that SRPX2 is expressed in language cortex, and *in utero* SRPX2 silencing impairs ultrasound vocalizations in mice.[16,17] The distinct effects of SRPX2 on neurodevelopmental processes make SRPX2 a favorable target for post-TBI tissue remodeling and repair.

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We recently reported a phylogenetically conserved neuronal expression of SRPX2 protein in the paraventricular (Pa), periventricular (Pe) and supraoptic (SO) nuclei of the hypothalamus.[19] We further demonstrated the colocalization of SRPX2 protein with oxytocin or vasopressin, and the presence of SRPX2 protein in rat and human plasma. However, very little is known about the factors influencing the expression of SRPX2 in the brain and plasma.

Here we investigated whether an acquired brain injury in an experimental model affecting the hypothalamus affects SRPX2 expression. We hypothesized that TBI-induced brain damage affects SRPX2 expression in the brain and that plasma SRPX2 levels can serve as a biomarker of TBI-induced hypothalamic injury. Our findings indicate that SRPX2 expression in the hypothalamus is drastically reduced acutely after severe TBI and that plasma SRPX2 levels correlate with the SRPX2 expression in the brain.

Materials and Methods

Animals

Two cohorts of adult (age 12-14 weeks, 320-450 g) male Sprague-Dawley rats (cohort 1, n=60, Harlan Laboratories, S.R.L., Italy [now Envigo Laboratories]; cohort 2, n=25, Envigo Laboratories, The Netherlands) were used in this study. Animals were housed in a controlled environment (temperature 22 ± 1°C, humidity 50%–60%, light–dark cycle from 07.00 to 19.00 h) with free access to food and water. All animal procedures were approved by the Animal Ethics Committee of the Provincial Government of Southern Finland, and performed in accordance with the guidelines of the European Community Council Directives 2010/63/EU.

Lateral Fluid-Percussion Injury

Cohort 1. Severe TBI was induced in 36 rats by lateral FPI as previously described in detail.[20,21] Briefly, animals were anesthetized with a cocktail (6 mL/kg, i.p.) of sodium pentobarbital (58 mg/kg), chloral hydrate (60 mg/kg), magnesium sulfate (127.2 mg/kg), propylene glycol (42.8%), and absolute ethanol (11.6%). The animals were placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) and the skull was exposed. A

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circular craniectomy (⌀ 5 mm) was performed over the left parietal lobe midway between lambda and bregma, leaving the dura intact. Lateral FPI (impact severity 3.33 ± 0.01 atm) was induced by connecting the rat to the fluid-percussion device (AmScien Instruments, Richmond, VA, USA). The duration of post-impact apnea and the occurrence of post-impact seizure-like behavior were recorded. Sham-operated animals (n=19) underwent the same procedure except for the fluid-percussion injury. Naive animals (n=5) without any treatment were also included in the study. Brain and blood samples were collected at 2 h, 6 h, 24 h, 48 h, 72 h, 5 d, 7 d, 14 d, 1 month and 3 months post-TBI.

Cohort 2. A validation cohort was included to evaluate the reproducibility of the reduction in plasma SRPX2 levels observed by Western blot in cohort 1. Severe TBI was induced in 13 rats by lateral FPI as described above. Briefly, animals were anesthetized with a cocktail (6 ml/kg, i.p.) of sodium pentobarbital (58 mg/kg), magnesium sulfate (127.2 mg/kg), propylene glycol (42.8%), and absolute ethanol (11.6%). Chloral hydrate was no longer allowed to be used as an anesthetic and was omitted from the anesthesia cocktail. Lateral FPI (impact severity 3.37 ± 0.24 atm) was induced by connecting the rat to the fluid-percussion device (AmScien Instruments). Duration of apnea and the occurrence of impact-induced seizure-like behavior were recorded. Sham-operated animals (n=8) underwent the same procedure except for the fluid-percussion injury. Naive animals (n=4) without any treatment were also included in the study. On the basis of data obtained from cohort 1, blood samples and brains were collected at 2 h post-TBI.

SRPX2 Western blot from rat plasma

Plasma Sampling. Rats were anesthetized with 5% isoflurane, and blood was drawn from the lateral tail vein as recommended by 3R (www.nc3rs.org.uk/the-3rs). In cohort 1, blood was also collected from the heart before perfusion using a 16G needle. The samples were centrifuged (1300g for 10 min), and the plasma was aliquoted and stored at -70°C.[22] To ensure the quality of the plasma used for Western blot analysis, hemoglobin was measured at 414 nm with a cutoff value of 0.25, as described previously.[22]

Western blot. The total protein concentration in the plasma was measured using a Pierce BCA (bicinchoninic acid) Protein Assay Kit (23225, Thermo Fisher Scientific). For Western blot analysis, plasma samples (5 µl initial plasma sample volume) were diluted

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with phosphate buffered saline to adjust the protein concentration to 2 µg/µl. The diluted plasma (20 µg protein), 1X Laemmli buffer, and β-mercaptoethanol were heated at 90^{o}C, separated on 12% TGX (Tris-Glycine eXtended) Stain-Free™ gels (Bio-Rad, Hercules, CA, USA) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. The membranes were blocked for 2 h in 1X TEN-1% Tween20 (10X TEN; 0.1 M Tris-Cl, 0.01 M EDTA, 1 M NaCl) solution and incubated overnight at 4^{o}C with rabbit polyclonal anti-SRPX2 (1:1000, ab91584, Abcam). The blots were then incubated with horseradish peroxidase goat anti-rabbit (1:10000, 65-6120, Invitrogen, Carlsbad, CA, USA) antibody for 1 h at room temperature. Immunoreactive bands were visualized using enhanced chemiluminescence substrate (SuperSignal™ West Pico Chemiluminescent Substrate, Thermo Fisher Scientific) on a gel documentation system (GelDoc Bio-Rad).

To obtain reliable quantitative data, we followed the guidelines recommended by Taylor and co-workers.[23] In view of limitations in reliable quantification using internal normalization proteins like β-actin, total protein-based normalization was used[24–26]. The linearity and limit of detection of SRPX2 protein by the SRPX2 antibody was determined. The antibody detected a quantifiable SRPX2-immunoreactive (ir) band in a serially diluted rat plasma sample containing ≥5 µg of total protein. To minimize the variation between blots, a replicate of the same naïve plasma sample was run in all gels as a positive control. As a negative control, Western blotting was repeated without the primary antibody and the SRPX2-ir band was not detected. Images were captured at all stages of the analysis, including (i) the activated stain-free gels after SDS-PAGE, (ii) the unstained blot after protein transfer and (iii) the SRPX2-immunostained blot. All images were acquired at the same exposure time and resolution, and analyzed using ImageJ software (version 1.51j8, National Institutes of Health, USA; www.imagej.nih.gov/ij).

For quantitative estimation of the plasma SRPX2 levels in plasma, the acquired images were converted to 8-bit gray scale. A region of interest (ROI) containing the SRPX2 band at 53 KDa was drawn and used on all blot images. For each SRPX2-immunostained blot, mean gray value (MGV) of the ROI containing the SRPX2 band was acquired. Background MGV from the respective blot was acquired using the same ROI in a band-free area and

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subtracted from the MGV of the ROI containing a SRPX2-ir band. The resultant MGV values were then normalized for total protein (as a loading control) and the positive control to minimize inter-membrane variability. Data are expressed as normalized band intensity ± SEM. The mean SRPX2 band intensity without normalization is presented in **Suppl. fig. 1**, indicating that total protein and positive control normalization did not affect the overall interpretation of the SRPX2 plasma levels after TBI.

Processing of brain tissue for histology

For immunohistochemistry, the rats were deeply anesthetized with a cocktail (6 mL/kg, i.p.) of sodium pentobarbital (58 mg/kg), magnesium sulfate (127.2 mg/kg), propylene glycol (42.8%), and absolute ethanol (11.6%). The animals were transcardially perfused with 0.9% saline (30 ml/min at 4°C) for 3 min followed by 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer, pH 7.4 (30 ml/min at 4°C), for 20 min. The brains were removed from the skulls and post-fixed in 4% PFA for 4 h (at 4°C), and then cryoprotected in a solution containing 20% glycerol in 0.02 M potassium phosphate buffered saline (KPBS) for 24 h. The brains were then frozen on dry ice and stored at -70°C until cut.

The brains were sectioned in a coronal plane (25 µm, in 1-in-12 series) with a sliding microtome (Leica SM 2000, Leica Microsystems Nussloch GmbH, Nussloch, Germany). The first series of sections was collected in 10% formalin at room temperature for thionin staining. The remaining series of sections were stored in tissue collecting solution (30% ethylene glycol, 25% glycerol in 0.05 M sodium phosphate buffer) at -20°C until staining.

Nissl staining

To identify the cytoarchitectonic boundaries of different brain areas and to optimize the selection of sections for analyses, the first series of sections was used for Nissl staining.[19] The sections were mounted on glass slides coated with 0.5% gelatin (G-2500, MilliporeSigma, Burlington, MA, USA), rehydrated in decreasing grades of alcohol, stained with thionin, dehydrated in increasing grades of alcohol, and cover-slipped with mounting medium (Depex® BDH Chemical, Poole, UK).

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Immunohistochemistry

One series of sections was stained for SRPX2 and oxytocin protein according to the immunohistochemistry protocol described earlier.[19] Briefly, sections were washed in 0.02 M KPBS (pH 7.4), incubated with 1% H_2O_2 to reduce the endogenous peroxidase activity and blocked in 10% normal goat serum and 0.5% Triton X-100. The sections were incubated in either rabbit polyclonal antibody raised against SRPX2 (1:10,000, ab91584, Abcam, Cambridge, UK) or rabbit anti-oxytocin antibody (1:16,000, AB911, MilliporeSigma) in KPBS containing 1% normal goat serum, and 0.5% Triton X-100 at 4°C for two nights. After washing with KPBS, the sections were then incubated for 1 h in biotinylated goat anti-rabbit IgG antibody (1:200, BA-1000, Vector Laboratories, Burlingame, CA, USA) followed by 1% avidin-biotin enzyme complex ABC (Vectastain® ABC kit, PK4000, Vector Laboratories) solution for 45 min. The antigen was visualized using 0.1% 3,3'-diaminobenzidine (Pierce Chemical, Rockford, IL, USA) solution containing 0.4% H_2O_2 in KPBS for 1 min. The sections were picked up on glass slides and dried overnight at 37°C. The staining reaction was intensified using 0.005% osmium tetroxide (OsO4, #19170, Electron Microscopy Sciences, Hatfield, PA, USA) as previously described.[27] Slides were covered with Depex® and dried overnight in the hood.

Estimation of the number of SRPX2-ir and oxytocin-ir neurons in the rat hypothalamus. SRPX2 and oxytocin immunoreactive cells throughout the rostrocaudal axis of the hypothalamus (1-in-12) were manually counted using ImageJ particle counter on bright-field photomicrographs acquired at 20X. The cytoarchitechtonic boundaries of the hypothalamic Pa, Pe and SO nuclei were identified prior to estimation of number of SRPX2-ir neurons. Photomicrographs of thionin-stained sections were captured and placed on top of SRPX2-immunostained photomicrographs using a transparent layer in Photoshop® (Adobe Photoshop CS5, version 12.0 x 64). The total number of SRPX2-ir cells (N_{tot}) was estimated according to the following formula: $N_{tot} = \sum Q \times 1/ssf$, where $\sum Q$ is the sum of cells counted from all sections and ssf is the section sampling fraction (1/12).[28]

Number of SRPX2-ir granules in SRPX2-ir neurons. To count the number of SRPX2-ir granules per neuron, 8-10 bright-field photomicrographs from the hypothalamic Pa and SO nuclei (bregma level -1.30 to -2.12 mm) were captured from two naïve and three 2 h post-

TBI rat brains using 100x objective lens, resulting in 2-3 SRPX2-ir cells per photomicrograph. The acquired images were converted to 8-bit gray scale. The number of immunolabeled granules per SRPX2-ir neuron was counted in the ipsilateral and contralateral Pa and SO nuclei.

Density of SRPX2-ir fibers in the hypothalamus. To estimate the density of SRPX2-ir fibers in the hypothalamus, two successive dark-field photomicrographs (275 µm apart, bregma -1.30 to -1.88) showing SRPX2-ir projections were captured from naïve (n=3) and 2 h post-TBI (n=3) rat brains using 10x objective lens. All images were captured at the same light intensity and acquisition settings. The acquired images were converted to 8-bit gray scale and threshold. The ROI containing the SRPX2-ir projections (ROI shown in **Fig. 6C**) was drawn on the ipsilateral and contralateral side of each section spanning the hypothalamic area between the Pa and SO nuclei and MGVs were acquired. Background was measured in each image from an area without the SRPX2-ir fibers and subtracted from the MGV acquired from ROI containing SRPX2-ir projections. Data are expressed as mean gray value per animal.

Statistical analysis

Data were analyzed using SPSS Statistics for Windows, ver. 25 (IBM, Armonk, NY, USA). Differences between the groups were analyzed using the Mann-Whitney U-test. Interhemispheric differences were analyzed using Wilcoxon's test. Correlations were assessed using Spearman's rho correlation coefficient. Receiver operating characteristics (ROC) analysis was performed to assess the sensitivity and specificity of plasma SRPX2 levels to discriminate rats with injury from naïve and sham-operated experimental controls. To investigate the possibility of identifying different sub-groups of animals clustered together on the basis of the number of SRPX2-ir neurons in the hypothalamus and SRPX2 plasma levels, we performed unsupervised hierarchical clustering in the R environment (version 3.0.1) (http//www.R-project.org/) using the Gplots package. Naïve rats, sham-operated experimental control rats and TBI rats were ordered using a clustering heat map with the single-linkage method together with the Manhattan distance measurement. Clusters were identified from the dendrograms. Variables included in the analysis were plasma SRPX2 level and number of SRPX2-ir neurons in hypothalamus,

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ipsilateral Pa, contralateral Pa, ipsilateral Pe, contralateral Pe, ipsilateral SO and contralateral SO. All data are expressed as mean ± SEM. A p-value of less than 0.05 was considered statistically significant.

Results

The number of animals used in the study and the overall study design is illustrated in Fig. 1 (A-B).

Impact severity, mortality, duration of post-impact apnea, and occurrence of acute post-impact seizure-like behavior

Despite the absence of chloral hydrate in anesthesia cocktail used in cohort 2, the outcome of both cohorts was comparable. For instance, the impact severity was comparable between cohort 1 and cohort 2 (3.33 ± 0.01 vs 3.37 ± 0.24 atm, $p>0.05$). The rate of acute mortality within 48 h post-TBI was 8 % (1 of 13 rats) in cohort 2. The mean duration of post-impact apnea was higher in cohort 1 (33.06 ± 2.8 s; range: 10-90 s) than that in cohort 2 (19.62 ± 3.10 s; range: 4-35 s) ($p<0.01$). Post-impact seizure-like behavior was observed in 38% (14 of 36) of rats in cohort 1 and 30% (4 of 13) of rats in cohort 2 ($p=0.60$, chi-square).

Plasma SRPX2 levels after TBI

We recently reported the presence of SRPX2 protein in rat and human plasma.[19] To investigate whether acquired brain injury affects SRPX2 expression, we analyzed the plasma SRPX2 levels in rats with lateral FPI-induced injury at 2 h, 6 h, 24 h, 48 h, 72 h, 5 d, 7 d, 14 d, 1 month, and 3 months post-TBI. Western blot analysis showed a marked decrease in the plasma SRPX2 levels at 2 h post-TBI compared with the sham-operated experimental controls (12.50 ± 5 vs 51.6 ± 3.18 arbitrary units (a.u.), $p<0.01$) and naïve rats (12.5 ± 5 vs 78.8 ± 13.4 a.u., $p<0.01$) **(Fig. 2A)**. The plasma SRPX2 levels were comparable between the sham-operated experimental controls and naïve rats ($p>0.05$).

At 6 h post-TBI, the plasma SRPX2 levels began to recover and the plasma SRPX2 levels were higher at 6 h-24 h post-TBI (average 46.83 ± 5.8 a.u.) than at 2 h post-TBI (12.50 ± 5 a.u., $p<0.01$). The plasma SRPX2 levels were higher between 48 h and 14 d post-TBI (43.76

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± 6.8 a.u.) than at 2 h post-TBI (12.50 ± 5 a.u., p<0.005). Within 1-3 months post-TBI, the plasma SRPX2 levels recovered completely and did not differ from those in naïve rats (78.64 ± 14.2 vs 78.89 ± 13.4 a.u., p>0.05) **(Fig. 2B)**. Ungrouped plasma SRPX2 levels of naïve, sham-operated experimental controls and TBI cases are presented in **Suppl. fig. 2**.

ROC analysis showed that SRPX2 plasma levels at 2 h post-TBI can differentiate between injured and sham-operated experimental controls (AUC= 1.00, p<0.05), and between injured and naïve rats (AUC=1.00, p<0.05) **(Fig. 3A-B)**.

Further analysis showed no correlations between the plasma SRPX2 levels and body weight, impact severity, or duration of post-impact apnea **(Suppl. table 2)**.

Spatiotemporal expression of SRPX2 protein after severe TBI

We have recently reported that SRPX2 protein is expressed in the hypothalamic Pa, Pe and SO nuclei of rat brain.[19] As the Western blot analysis showed a reduction in plasma SRPX2 levels at 2 h post-TBI, we investigated if this change correlated with the SRPX2 expression in brain after TBI. Therefore, we performed quantitative estimation of the SRPX2-ir neurons in the hypothalamus at 2 h, 6 h, 24 h, 48 h, 24 h, 48 h, 72 h, 5 d, 7 d, 14 d and 1 month post-TBI **(Fig. 4)**. The mean SRPX2-ir neuronal counts at each time point are presented in **Suppl. table 1.**

SRPX2-ir neuronal numbers in the hypothalamus at 2 h post-TBI

Stereological cell counting revealed a reduction in the total number of SRPX2-ir neurons in the hypothalamic Pa (2076 ± 386 vs 4344 ± 247, p<0.05) and SO (2468 ± 202 vs 4532 ± 419, p<0.05) nuclei at 2 h post-TBI compared with that in the naïve group **(Fig. 4A, 6A)**. At 2 h post-TBI, the number of SRPX2-ir neurons was reduced to 49% in the Pa (p<0.001) **(Suppl. table 1),** 86% **in the** Pe, and 54% in the SO of that in naive rats **(Suppl. table 1)**. SRPX2-ir neuronal numbers were comparable between ipsilateral and contralateral Pa, Pe and SO in naive and 2 h post-TBI rats (p>0.05). Taken together, the total number of SRPX2-ir neurons in the hypothalamus at 2 h post-TBI (5032 ± 527 vs 9440 ± 351) was 53% of that in the naïve rats. The number of SRPX2-ir neurons did not differ between the sham-operated experimental controls and the naïve group (p>0.05) **(Fig 4A-B)**.

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Distribution of SRPX2-ir neurons in the Pa nucleus. The number of SRPX2-ir neurons was decreased bilaterally at 2 h post-TBI. Within the Pa nucleus, the decrease was more pronounced in neurons of the dorsal cap of Pa (PaDC), lateral magnocellular Pa (PaLM), medial parvocellular Pa (PaMP) and ventral part of Pa (PaV) (bregma level -1.80 to -1.92 mm) **(Fig 5A-B)**. The number of SRPX2-ir neurons in the anterior parvocellular Pa (PaAP), medial magnocellular Pa (PaMM) (bregma level -0.96 to -1.60 mm), and posterior Pa (PaPo) (bregma level -2.04 mm) was comparable between the naïve and 2 h post-TBI groups ($p>0.05$) **(Fig 5A)**.

Number of SRPX2-ir granules per SRPX2+ neuron. Cytoplasmic SRPX2 protein has a granular appearance under higher magnification. We investigated whether the number of SRPX2-ir granules per neuron differed between the naïve and 2 h post-TBI group **(Fig. 6B)**. The number of SRPX2-ir granules per SRPX2-ir neuron was counted in the ipsilateral and contralateral Pa (n=14 SRPX2-ir neurons per rat, 7 from each hemisphere) and SO (n=14 SRPX2-ir neurons per rat, 7 from each hemisphere) nuclei. The number of SRPX2-ir granules/cell was reduced in the ipsilateral Pa at 2 h post-TBI (62.9 ± 2.6 vs 71.3 ± 3.5, $p<0.05$) compared with the naïve group.

Density of SRPX2-ir fibers at 2 h post-TBI. SRPX2-ir fibers from the Pa and SO neurons travel ventrally toward the hypophysis. We investigated whether there is an injury-induced difference in the SRPX2-ir fiber density at 2 h post-TBI **(Fig. 6C)**. The density of SRPX2-ir fibers on the ipsilateral side of the hypothalamus of the injured rats did not differ from that on the contralateral side (37.8 ± 11.8 vs 34.6 ± 7.7, $p>0.05$). Moreover, the mean ipsilateral SRPX2-ir fiber density at 2 h post-TBI (37.8 ± 11.8 vs 53.3 ± 12.5, $p>0.05$) was comparable with that of the naive group **(Fig. 6C)**.

SRPX2-ir neuronal numbers in the hypothalamus at 6 h - 24 h post-TBI

At 6 h post-TBI, SRPX2-ir neuronal numbers were 84% and 78% of that in naive Pa and SO nuclei, respectively. The total number of SRPX2-ir neurons in the hypothalamus was comparable between naïve and 24 h post-TBI (99% of naïve) **(Fig. 4, Suppl. table 1)**.

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SRPX2-ir neuronal numbers in the hypothalamus at 48 h - 1 month post-TBI

The number of SRPX2-ir neurons was also decreased at 48 h post-TBI, lasting up to 14 d post-TBI. During this time window, SRPX2 expression varied between animals at the same time point. At 1 month post-TBI, the number of SRPX2-ir neurons in the hypothalamus was 97% of that in the naïve rats **(Fig. 4, Suppl.table 1)**.

Further analysis showed no significant correlation between the total number of SRPX2-ir neurons in the hypothalamus after TBI and body weight, impact severity, or post-impact apnea **(Suppl. table 2)**.

Comparison of TBI-induced changes in SRPX2 expression in plasma and brain

When all TBI groups were pooled together, the plasma SRPX2 levels showed an overall correlation with the number of hypothalamic SRPX2-ir neurons ($r=0.46$, $p<0.05$) **(Fig 7A)**. The plasma SRPX2 levels and SRPX2-ir neuronal numbers, however, did not correlate in all cases at 6-24 h and 2-14 d post-TBI group. The number of SRPX2-ir neurons in the hypothalamus strongly correlated with the plasma SRPX2 level in the same animals at 2 h post-TBI ($r=0.89$, $p<0.001$) **(Fig. 7B)**. Also, the number of SRPX2-ir neurons in the hypothalamus strongly correlated with the plasma SRPX2 level at 1 month post-TBI ($r=0.94$, $p<0.001$**) (Fig 7B).**

Unsupervised hierarchical clustering was applied to the SRPX2 protein expression data obtained by Western blot and immunohistochemistry (cohort 1) to investigate whether animals from uninjured and injured groups can be differentiated into clusters based on the number of SRPX2-ir neurons in the Pa, Pe, SO and total hypothalamus or the plasma SRPX2 levels. No assumptions were made in the clustering analysis and thus the “unsupervised” analysis determined the relatedness of cases based on the pattern of SRPX2 protein expression in brain or plasma irrespective of the experimental group.

Unsupervised hierarchical clustering utilizing the number of SRPX2-ir neurons in the Pa, Pe and SO nuclei, and the hypothalamus on the whole, differentiated rats into two main clusters. The 2 h, 6 h, 72 h, 5 d, 7 d and 14 d post-TBI groups were clustered together in

cluster 1. The 24 h, 48 h and 1 month post-TBI groups were clustered with naïve and sham groups in cluster 2 (Fig 8A).

Further analysis using unsupervised hierarchical clustering utilizing the plasma SRPX2 levels and number of SRPX2-ir neurons in the hypothalamus identified two main clusters. The 2 h, 6 h, 72 h, 5 d, 7 d and 14 d groups were clustered in cluster 1. In cluster 2, the 24 h, 48 h and 1 month post-TBI groups were clustered with naïve and sham-operated experimental control animals (Fig 8B).

Oxytocin expression in the hypothalamus at 2 h post-TBI

We have previously demonstrated that SRPX2 colocalized with oxytocin in the Pa and SO nuclei of the hypothalamus.[19] Therefore, to investigate whether the reduction in the number of SRPX2-ir neurons correlated with oxytocin-ir neuronal numbers, we performed oxytocin immunolabeling in brain sections obtained from the naïve and 2 h post-TBI groups. Similar to SRPX2, the total number of oxytocin-ir neurons in the hypothalamus was markedly decreased in the 2 h post-injury group compared with the naïve group (4780 ± 794 vs 8408 ± 278, $p<0.001$) **(Fig 9A)**. Stereological cell counting revealed a reduction in the total number of oxytocin-ir neurons in the hypothalamic Pa at 2 h post-TBI (2708 ± 685 vs 5436 ± 138, $p<0.05$) compared with the naïve group **(Fig 9B)**. The total number of oxytocin-ir neurons in the SO nucleus (2072 ± 122 vs 2972 ± 203) was comparable between the 2 h post-TBI and naïve group **(Fig 9B)**. Moreover, the number of oxytocin-ir neurons in the hypothalamus strongly correlated ($r=0.99$, $p<0.01$) with the number of SRPX2-ir neurons in the same animals at 2 h post-TBI **(Fig 9C)**.

SRPX2 expression in the validation cohort at 2 h post-TBI

To ensure reproducibility of the observed downregulation of SRPX2 expression in the plasma and brain at 2 h post-TBI, we performed Western blot and immunohistochemical analysis in a validation cohort. To enhance the statistical power of the study, the sample size for cohort 2 was estimated by computing the effect size from cohort 1 (G*Power v 3.1.9.4 for windows). Similar to cohort 1, Western blot analysis in cohort 2 showed a marked decrease in the plasma SRPX2 levels at 2 h post-TBI as compared with the sham-operated control and naïve groups **(Fig. 10)**. SRPX2 immunohistochemical analysis in these

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animals also showed reduction in SRPX2 immunoreactivity in the hypothalamus. These observations validated the findings from cohort 1.

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Discussion

In the present study we investigated whether TBI affects the expression of SRPX2 protein in rat plasma, and if these TBI-induced changes correlate with the number of SRPX2-ir neuron in the brain. We hypothesized that SRPX2 expression changes after TBI and SRPX2 plasma levels can serve as a diagnostic biomarker for hypothalamic injury. We identified a reduction in plasma SRPX2 levels at 2 h post-TBI that correlated with number of SRPX2-ir neurons in the hypothalamus at 2 h post-TBI.

SRPX2 is a novel biomarker of TBI-induced hypothalamic injury

In humans, hypothalamic injury is not uncommon, and impairments in hypothalamic connectivity are reported after TBI.[29,30] Moreover, studies in humans and experimental models report hypopituitarism and an impaired HPA axis after traumatic brain injury.[8–11] The lack of hypothalamic injury-specific biomarkers, however, limits the detection of TBI-induced endocrinopathy.

We have recently showed SRPX2 expression in the hypothalamus and its presence in rat and human plasma.[19] The well-defined effects of SRPX2 on synaptic plasticity, angiogenesis, and tissue remodeling[15,31,32] make SRPX2 a promising target to modulate tissue repair after brain injury or disease. Therefore, we investigated the expression of SRPX2 in the hypothalamus and plasma at acute and chronic time points after acquired brain injury.

We found that the plasma SRPX2 levels are reduced at 2 h after TBI and this reduction correlates with the number of SRPX2-ir neurons in the hypothalamus, indicating potential brain-specific regulation of SRPX2 expression after acquired brain injury. The reduction in hypothalamic SRPX2-ir neurons at 2 h post-TBI was bilateral. Considering the location of the hypothalamo-pituitary axis at the bottom of the skull at midline, it can be suggested that the impact force has a bilateral effect on the brain as previously shown in human TBI patients.[33] The reduced number of SRPX2-ir cytoplasmic granules in SRPX2-ir neurons of Pa nucleus at 2 h post-TBI indicates possible downregulation of the vesicular packaging of SRPX2 protein, consequently downregulating SRPX2 secretion to the plasma.

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In naïve rat brain, SRPX2-ir neurons were present in all three functional compartments of Pa nucleus including the magnocellular neurons, the neuroendocrine parvocellular neurons and the pre-autonomic parvocellular neurons.[19] At 2 h post-TBI, the decrease in the number of SRPX2-ir neurons in the hypothalamus was bilateral and most visible in the Pa nucleus including both the magnocellular and parvocellular subdivisions. The downregulation of SRPX2 expression can, therefore, have implications in regulation of both endocrine and autonomic functions of hypothalamo-pituitary axis after an injury to the brain. Damage to the hypothalamic Pa is documented previously in the lateral FPI model of TBI. For example, bilateral upregulation of corticotropin-releasing hormone (CRH) mRNA is observed in the hypothalamic Pa two hours after FPI.[34] Furthermore, post-traumatic hyperthermia in rats 7 days after lateral FPI is associated with increased astrocytosis and inflammation in the hypothalamic Pa.[35] These studies together with our data indicate that the hypothalamus is vulnerable to TBI-induced damage and the lateral FPI model of TBI recapitulates hypothalamic injury in humans.

The recovery of SRPX2 plasma levels at 1 month post-TBI also correlated with the number of SRPX2-ir neurons in the brain. The variable expression of SRPX2 between 2 d to 14 d after injury may be attributed to ongoing temporal evolution of injury-induced processes and underlying molecular networks.[36] Interestingly, our preliminary data show that plasma SRPX2 levels measured at 2 d after the 2nd and 4th hit in the repeated mild TBI model were comparable with those in naïve animals, whereas 2 d after a single severe TBI, the plasma SRPX2 levels in 50% of animals were less than -1SD from the mean of the naïve group. Repeated impact, however, caused a 30% reduction in the number of hypothalamic SRPX2-ir neurons when assessed at 2 weeks after the last impact **(see Supplementary figure 3)**. These data suggest a potential impact-dose effect on plasma SRPX2 levels. Further, although not measurable in plasma by Western blot analysis, repeated mild TBI can downregulate hypothalamic SRPX2 expression.

Unsupervised hierarchical clustering analysis identified injury-specific clusters based on SRPX2 protein expression in the brain and plasma. This analysis indicated that SRPX2 expression is differentially regulated in rats after lateral FPI. However, the 24 h, 48 h and 1 month post-TBI groups clustered with naïve and sham-operated experimental control groups, indicating that the SRPX2 expression in the brain and plasma at these time points

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was comparable between controls and TBI. Taken together, these data indicate an injury-specific downregulation in SRPX2 expression after TBI and it can be proposed that SRPX2 protein may serve as a candidate biomarker of hypothalamic injury. It is however yet to be explored if plasma SRPX2 levels can serve as a clinical biomarker of hypothalamic injury. Human studies investigating temporal evolution of TBI-induced changes in plasma SRPX2 levels will evaluate the translational capacity of our findings. However, the current study provides a starting point for exploring the molecular mechanisms that regulate SRPX2 expression after an insult to the brain.

Effect of TBI on oxytocin immunoreactivity in hypothalamus

SRPX2 protein colocalizes with oxytocin or vasopressin in the hypothalamus.[19] Similar to SRPX2, the total number of neurons expressing oxytocin in the Pa and SO nuclei was decreased at 2 h after TBI. Oxytocin plays an important role in social communication and language.[37–40] Moreover, oxytocin has been identified as a stress buffer in mammalian stress response.[41] Acute intranasal oxytocin administration attenuates chronic pain in rats after lateral FPI.[42] The reduction in the number of oxytocin-ir neurons in the paraventricular nucleus further emphasizes the TBI-induced hypothalamic damage.

Conclusion

Taken together, our data indicate that SRPX2 is a candidate biomarker of hypothalamic injury. The rapid downregulation of SRPX2 protein following lateral FPI suggests that SRPX2 is involved in the pathogenesis of TBI and may have important implications for the development of novel therapeutic strategies for treating TBI-associated pathology and comorbidities.

Acknowledgments / Role of authors

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Author contributions

M.A., X.N., J.K., N.P., and A.P. designed the research

M.A., and L.L. performed the research

M.A., X.N., N.P., J.K. and A.P. analyzed the data

M.A. and A.P. wrote the paper with input from all the authors

Author Disclosure Statement

No competing financial interests exist.

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Figure 1

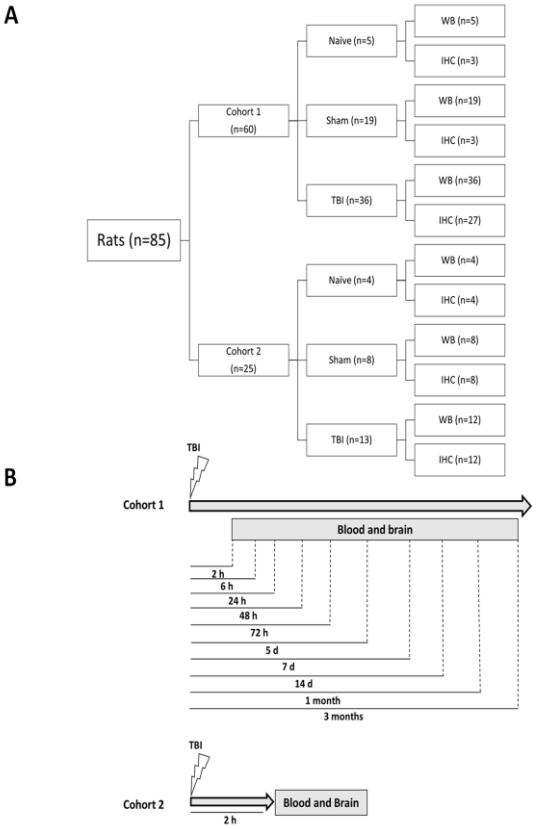

Figure 1. Schematic illustration of the study design. (A) The number of animals used in each cohort for Western blot (WB) and immunohistochemical (IHC) analysis. **(B)** In cohort 1, blood and brain samples were collected at 2 h, 6 h, 24 h, 48 h, 72 h, 5 d, 7 d, 14 d, 1 month, and 3 months post-TBI. Cohort 2 validated the changes in SRPX2 protein expression at 2 h post-TBI.

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Figure 2

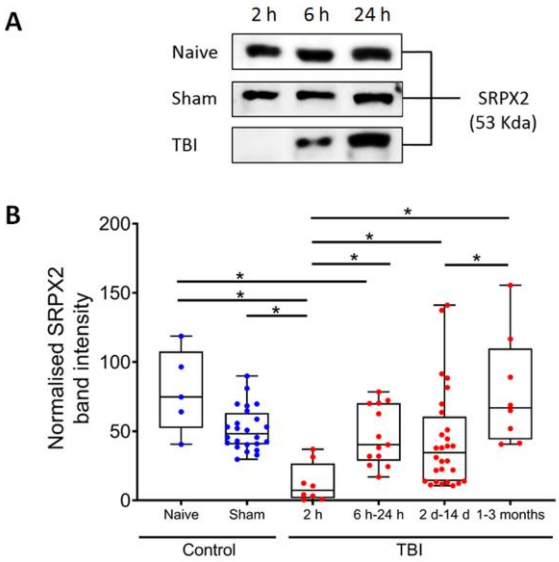

Figure 2. Plasma SRPX2 levels after severe TBI. **(A)** SRPX2 plasma levels decreased markedly at 2 h post-TBI compared with those in naïve and sham-operated experimental controls. **(B)** Plasma SRPX2 levels began to recover at 24 h post-TBI, reaching the level of those in naïve and sham-operated experimental controls at 1-3 months post-TBI. Note the great variability in plasma SRPX2 levels in the 2 d-14 d group, in which plasma SRPX2 levels in a large number of animals were still below those in naïve rats or sham-operated experimental controls. SRPX2 plasma levels were determined by Western blot analysis, where equal amounts of total protein (20 µg) were loaded in all wells. SRPX2 band intensity was normalized with total protein (as a loading control) and a positive control within the same gel. Data are expressed as mean ± SEM (arbitrary units, a.u.). * = p-value <0.05.

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Figure 3

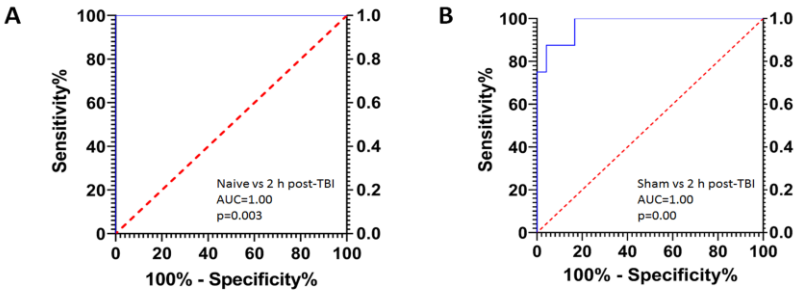

Figure 3. Receiver operating characteristics (ROC) analysis of plasma SRPX2 levels after severe TBI. ROC analysis showing the sensitivity and specificity of plasma SRPX2 levels at **(A)** 2 h post-TBI (n=8) vs. naïve (n=5) and **(B)** 2 h post-TBI (n=8) vs. sham-operated experimental controls (n=24) to differentiate between injured and control rats.

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Figure 4

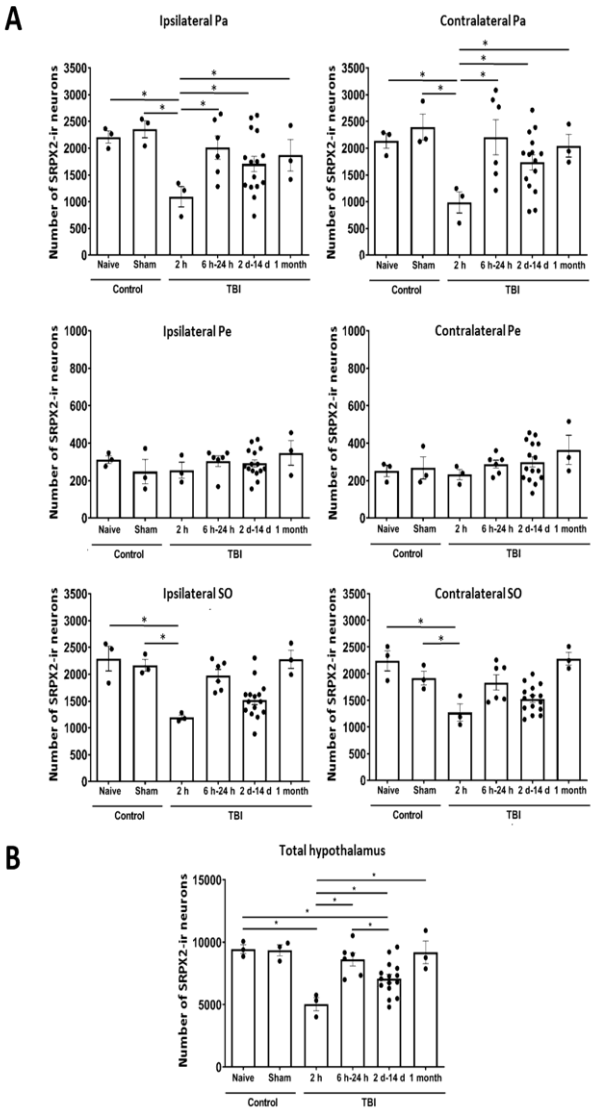

Figure 4. SRPX2-ir neurons in the hypothalamus after severe TBI. (A) The number of SRPX2-ir neurons decreased in the ipsilateral and contralateral Pa and SO nuclei at 2 h after injury. **(B)** The total number of SRPX2-ir neurons in the hypothalamus was strikingly reduced at 2 h post-TBI. At 1 month post-TBI, the total number of SRPX2+ neurons was comparable with that in the naïve and sham groups.

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Figure 5

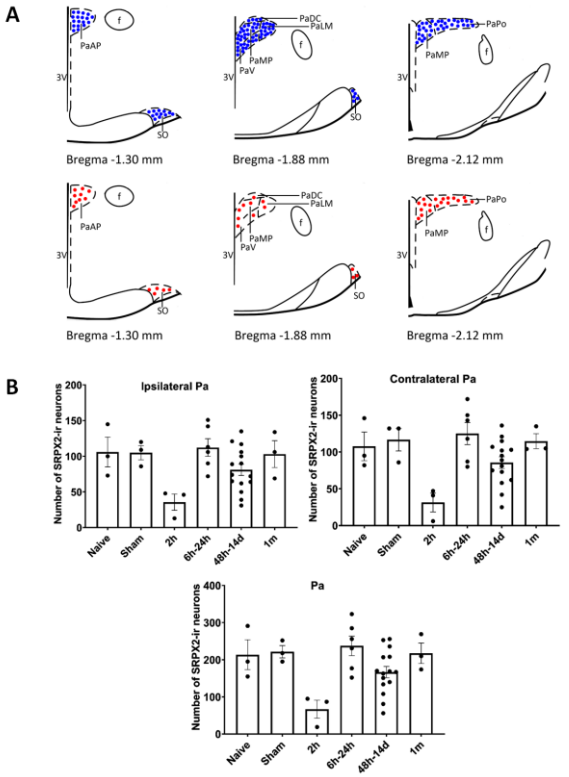

Figure 5. Distribution of SRPX2-ir neurons in the hypothalamic paraventricular (Pa) and supraoptic (SO) nuclei at 2 h after severe TBI. (A) A schematic illustration showing the distribution of SRPX2-ir neurons in the hypothalamus in naïve (in blue) and injured (in red) rat brain at 2 h post-TBI [adapted from the rat brain atlas of Paxinos and Watson (2007)] depicting a profound overall reduction in SRPX2-ir neurons in the Pa at bregma level -1.88mm. **(B)** In the 2 h post-TBI group, the number of SRPX2-ir neurons at bregma level -1.88 mm in all animals was less than that in the control group (<-1SD from naive mean). Abbreviations: Pa, paraventricular nucleus; PaDC, Pa dorsal cap; PaLM, Pa lateral magnocellular; PaMP, Pa medial parvocellular; PaV, Pa ventral part; PaAP, Pa anterior parvocellular; PaMM, Pa medial magnocellular; PaPo, Pa posterior.

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Figure 6

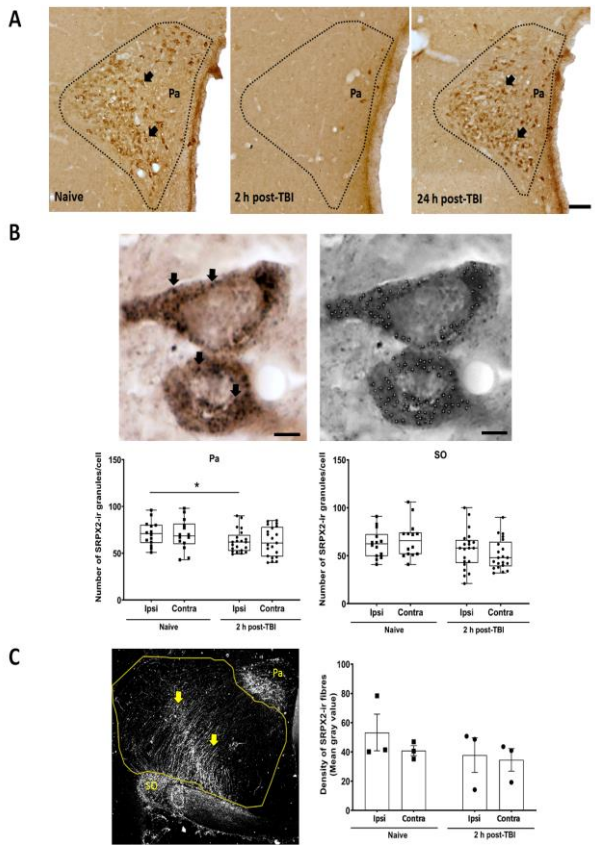

Figure 6. SRPX2 expression in brain was decreased at 2 h post-TBI and associated with a lower number of SRPX2-ir granules per neuron. (A) At 2 h post-TBI, the number of SRPX2-ir neurons (black arrows) was strikingly lower in the Pa nucleus (ipsilateral shown) compared with that in naïve rats ($p<0.05$). At 24 h post-TBI, the number of SRPX2-ir neurons was no longer different from that in the naïve group ($p>0.05$). **(B)** Higher magnification photomicrographs of SRPX2-ir neurons showing SRPX2-ir granules (black arrows) were converted to 8-bit gray scale and SRPX2-ir granules per cell were counted. At 2 h post-TBI, TBI rats had 11% fewer SRPX2-ir granules per neuron in the Pa nucleus than the naïve animals ($p<0.05$). The number of SRPX2-ir granules in the SO nucleus, however, was comparable with that in the naïve group ($p>0.05$). **(C)** Dark-field photomicrograph of the ipsilateral hypothalamus in a naïve rat showing SRPX2-ir axonal (yellow arrows) density (ROI shown in yellow). Bar graph shows that the density of SRPX2-ir axons in the 2 h post-TBI group was similar to that in the naïve control group ($p>0.05$). Abbreviations: ipsi, ipsilateral; contra, contralateral; Pa, paraventricular nucleus; SO, supraoptic nucleus. Scale bar equals (A) 50 µm and (B) 5 µm.

Figure 7

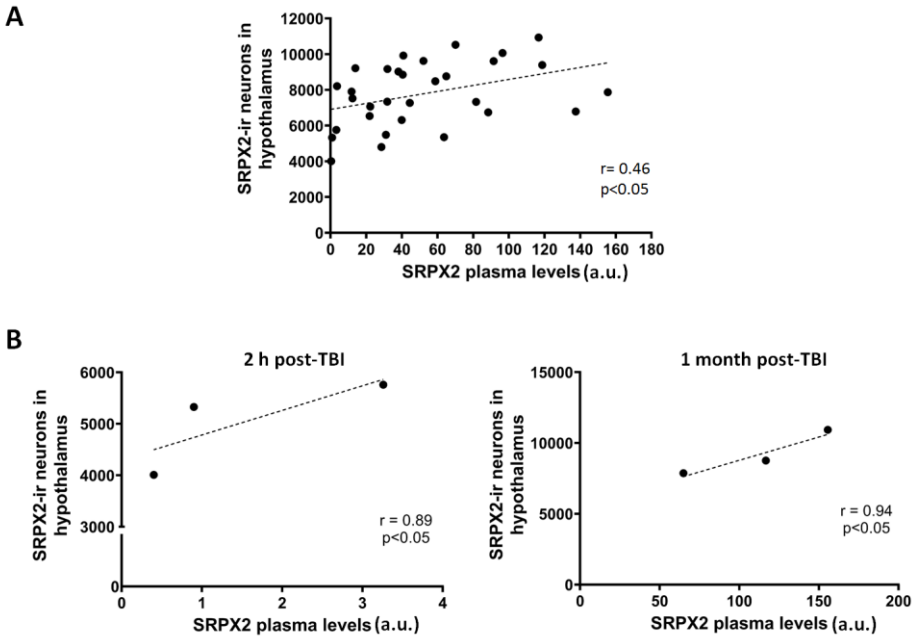

Figure 7. Correlation of SRPX2 expression in the plasma and brain. (A) Correlation of plasma SRPX2 levels with the number of SRPX2-ir neurons in the hypothalamus of the TBI group (2 h-1 month). **(B)** Plasma SRPX2 levels correlated with the total number of SRPX2-ir neurons in the hypothalamus at 2 h (left) and at 1 month (right) post-TBI. Abbreviations: a.u., arbitrary units; ir, immunoreactive.

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Figure 8

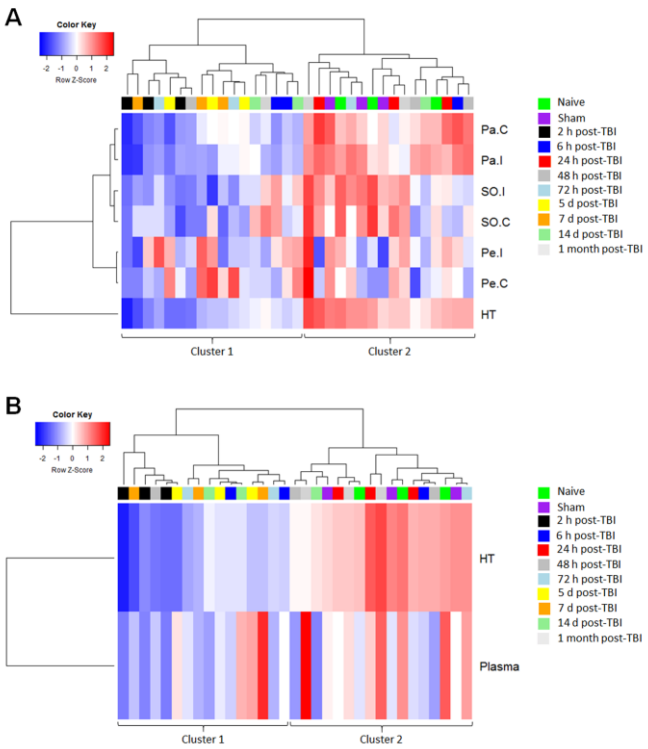

Figure 8: Unsupervised hierarchical clustering of the three animal groups (naïve, sham-operated experimental controls, TBI) based on the number of SRPX2-ir neurons in the different hypothalamic nuclei ipsilaterally and contralaterally and the plasma SRPX2 levels in the same rats. **(A)** Based on number of SRPX2-ir neurons in the Pa, Pe, SO nuclei or total hypothalamus, rats were differentiated into two main clusters, injured and uninjured. However, 3 TBI rats in the 24 h, 2 in the 48 h and 2 in the 1 month post-TBI groups clustered with controls. Each column represents an individual animal, and each row represents the cell counts in the ipsilateral and contralateral Pa, Pe, SO nuclei and the total hypothalamus. **(B)** The number of SRPX2-ir neurons in the hypothalamus and the plasma SRPX2 levels also identified two main clusters. Each column represents an individual animal, and each row represents the SRPX2-ir neuronal numbers in hypothalamus and plasma SRPX2 levels. Colors in the heatmap represent variable values as a Z-score: Higher = red, lower = blue. Abbreviations: I, ipsilateral; C, contralateral; HT, hypothalamus; Pa, paraventricular; Pe, periventricular; SO, supraoptic.

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Figure 9

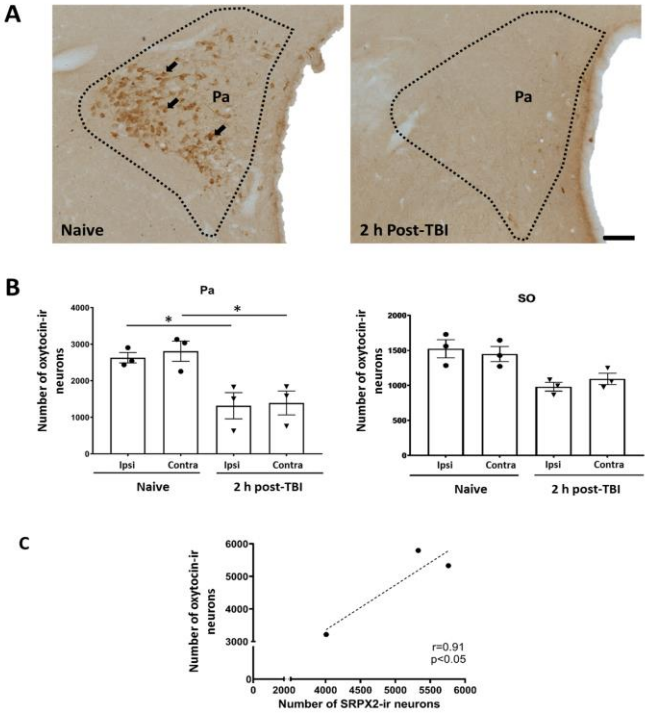

Figure 9. Oxytocin expression in the hypothalamus at 2 h post-TBI. (A) Oxytocin-ir neurons in the Pa nucleus of naïve and 2 h post-TBI rats. **(B)** The number of oxytocin-ir neurons in the ipsilateral and contralateral Pa and SO decreased 2 h after injury compared with naïve animals. **(C)** The number of SRPX2-ir neurons in the hypothalamus correlated with number of oxytocin-ir neurons in the hypothalamus at 2 h post-TBI. Abbreviations: contra, contralateral; Ipsi, ipsilateral; ir, immunoreactive; Pa, paraventricular nucleus, SO, supraoptic nucleus. Scale bar equals 50 µm.

Figure 10

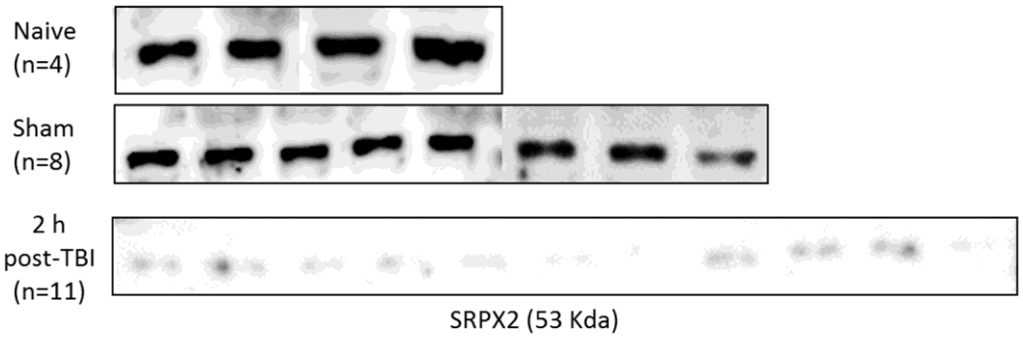

Figure 10. Plasma SRPX2 levels in naïve, sham-operated experimental controls, and 2 h post-TBI rats from the validation cohort. The SRPX2-ir band intensity decreased prominently at 2 h post-TBI, indicating downregulation of plasma SRPX2 levels acutely after TBI.

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SRPX2 expression after repeated mild TBI

Induction of repeated mild TBI (rmTBI). In order to investigate the injury-dose effect on hypothalamic SRPX2-ir neurons and plasma SRPX2 levels, we induced rmTBI using lateral fluid-percussion injury (lateral FPI) in 5 rats **(see Suppl. fig. 3A)**. Briefly, animals were anesthetized with isofluorane (4% for induction, 1.5-2.4% during surgery). The animals were placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) and the skull was exposed. A circular craniectomy (⌀ 5 mm) was performed over the left parietal lobe midway between lambda and bregma, leaving the dura intact. Lateral FPI (impact severity 1.39 ± 0.04 atm) was induced by connecting the rat to the fluid-percussion device (AmScien Instruments, Richmond, VA, USA). Rats were removed from the device and the scalp was sutured. Buprenorphine (0.05 mg/kg) was administered for postoperative analgesia. The same procedure was performed three more times at 1-week intervals following the 1st rmTBI.

Composite neuroscore. Motor function was assessed with the composite neuroscore 1 d before first impact and 2 d after every rmTBI. The assessment was performed as previously described.[39] Briefly, animals were scored for forelimb flexion (right and left separately), hindlimb flexion (right and left separately), and resistance to right and left lateral pulsion, and the ability to maintain position on an inclined plane (maximum score 28). The composite neuroscore decreased at 2 d after the first mild TBI by 2 points ($p>0.05$), but did not worsen with repeated injuries **(Suppl. fig. 3B)**.

Plasma SRPX2 levels. To investigate the changes in expression of SRPX2 after rmTBI, plasma samples (n=5) were collected two days after the 2nd and 4th hit for Western blot analysis. Brain tissue (n=4) was collected at 14 days after the 4th rmTBI for SRPX2 immunohistochemistry. Plasma SRPX2 levels after the 2nd and 4th hit were comparable with plasma SRPX2 levels of naïve animals from cohort 1 **(Suppl. fig. 3C)**.

Hypothalamic SRPX2 immunoreactive neuronal numbers in rats with rmTBI. Next, we investigated the effect of rmTBI on SRPX2 expression in the brain **(Suppl. table 3, Suppl. fig 3D-G)**. At 14 d after the 4th hit (i.e., 5 weeks after the 1st hit), the number of SRPX2-ir neurons in the ipsilateral Pa was 74% of that in naïve animals (3 195 ± 310 vs 4 344 ± 247,

p<0.05) **(Suppl. fig. 3D)**. The total number of hypothalamic SRPX2-ir neurons was 70% of that in naïve controls (6 650 ± 484 vs 9 440 ± 351, p<0.05) **(Suppl. fig. 3G)**.

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Supplementary data

	Pa			Pe			SO			Pa + Pe + SO
	Ipsilateral	Contralateral	Total	Ipsilateral	Contralateral	Total	Ipsilateral	Contralateral	Total	Hypothalamus
Naive	2208 ± 111	2136 ± 138	4344 ± 247	312 ± 12	252 ± 30	564 ± 50	2292 ± 230	2240 ± 190	4532 ± 419	9440 ± 351
Sham	2352 ± 157 (106%)	2392 ± 244 (111%)	4744 ± 367 (109%)	248 ± 64 (79%)	268 ± 59 (106%)	516 ± 123 (91%)	2164 ± 107 (94%)	1920 ± 129 (85%)	4084 ± 235 (90%)	9344 ± 439 (98%)
2 h	1092 ± 190 (49%)	984 ± 196 (46%)	2076 ± 386 (47%)	256 ± 42 (82%)	232 ± 28 (128%)	488 ± 61 (86%)	1196 ± 46 (52%)	1272 ± 161 (56%)	2468 ± 202 (54%)	5032 ± 527 (53%)
6 h	1792 ±378 (81%)	1880 ± 520 (88%)	3672 ± 898 (84%)	328 ± 11 (105%)	272 ± 29 (107%)	600 ± 39 (106%)	1856 ± 177 (80%)	1708 ± 203 (76%)	3564 ± 380 (78%)	7836 ± 674 (83%)
24 h	2228 ± 229 (100%)	2528 ± 410 (118%)	4756 ± 629 (109%)	280 ± 56 (89%)	304 ± 35 (120%)	584 ± 88 (103%)	2104 ± 123 (91%)	1960 ± 223 (87%)	4064 ± 334 (89%)	9404 ± 570 (99%)
48 h	2084 ± 397 (93%)	2036 ± 410 (95%)	4120 ± 801 (94%)	276 ± 7 (88%)	184 ± 26 (73%)	460 ± 21 (81%)	1504 ± 158 (65%)	1452 ± 183 (64%)	2956 ± 289 (65%)	7536 ± 1093 (79%)
72 h	1868 ± 375 (84%)	1824 ± 348 (85%)	3692 ± 717 (84%)	320 ± 51 (102%)	332 ± 66 (131%)	652 ± 22 (115%)	1728 ± 306 (75%)	1592 ± 76 (71%)	3320 ± 382 (73%)	7664 ± 998 (81%)
5 d	1404 ± 235 (63%)	1548 ± 354 (72%)	2952 ± 547 (67%)	324 ± 37 (103%)	372 ± 57 (147%)	696 ± 93 (123%)	1268 ± 212 (55%)	1540 ± 166 (68%)	2808 ± 87 (61%)	6456 ± 573 (68%)
7 d	1276 ± 294 (57%)	1496 ± 343 (70%)	2772 ± 631 (63%)	252 ± 79 (80%)	308 ± 70 (122%)	560 ± 144 (99%)	1400 ± 46 (61%)	1312 ± 138 (58%)	2712 ± 103 (59%)	6044 ± 626 (64%)
14 d	1892 ± 269 (85%)	1768 ± 271 (82%)	3660 ± 536 (84%)	288 ± 37 (92%)	300 ± 48 (119%)	588 ± 84 (104%)	1712 ± 163 (74%)	1728 ± 136 (77%)	3440 ± 209 (75%)	7688 ± 266 (81%)
1 month	1868 ± 296 (84%)	2044 ± 210 (95%)	3912 ± 506 (90%)	348 ± 66 (111%)	364 ± 79 (144%)	712 ± 143 (126%)	2280 ± 170 (99%)	2284 ± 119 (101%)	4564 ± 265 (100%)	9188 ± 909 (97%)

% of naive (corresponding side and nucleus)

Supplementary table 1. Spatiotemporal hypothalamic SRPX2 expression after severe lateral FPI. Table showing total number of SRPX2 immunoreactive neurons in the hypothalamic paraventricular (Pa), periventricular (Pe) and supraoptic (SO) nuclei of the

rat brain after traumatic brain injury (3 rats per group). No statistically significant interhemispheric differences were identified. Note the reduction in number of SRPX2+ neurons in hypothalamus at 2 h post-TBI. Data are expressed as mean ± SEM.

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Supplementary table 2. Table showing correlation of post-impact severity, apnea, and body weight with SRPX2 expression.

Comparison		Correlation (number of animals)
At 2 h post-TBI	Impact severity vs plasma SRPX2 levels	r=0.71, p=0.057 (n=8)
	Post-impact apnea vs plasma SRPX2 levels	r=0.26, p=0.52 (n=8)
	Body weight vs plasma SRPX2 levels	r=0.61, p=0.11 (n=8)
	Post-impact apnea vs impact severity	r=0.37, p=0.36 (n=8)
	Post-impact apnea vs body weight	r=0.08, p=0.84 (n=8)
	Impact severity vs number of SRPX2+ neurons in hypothalamus	r=0.50, p>0.99 (n=3)
	Impact severity vs number of SRPX2+ neurons in Pa nucleus	r=1.00, p=0.33 (n=3)
In TBI group (all time points together)	Impact severity vs number of SRPX2+ neurons in hypothalamus	r=-0.02, p=0.91 (n=27)
	Impact severity vs number of SRPX2+ neurons in Pa nucleus	r=-0.04, p=0.81 (n=27)
	Body weight vs number of SRPX2+ neurons in Pa nucleus	r=0.16, p=0.42 (n=27)
	Body weight vs number of SRPX2+ neurons in hypothalamus	r=0.26, p=0.18 (n=27)
	Post-impact apnea vs number of SRPX2+ neurons in hypothalamus	r=0.04, p=0.83 (n=27)
	Impact severity vs post-impact apnea	r=-0.07, p=0.65 (n=36)

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	Paraventricular Nucleus (Pa)			Periventricular Nucleus (Pe)			Supraoptic Nucleus (SO)			Pa + Pe +SO
Group	Ipsilateral	Contralateral	Total Pa	Ipsilateral	Contralateral	Total Pe	Ipsilateral	Contralateral	Total SO	Hypothalamus
Naive (n=3)	2208 ± 111	2136 ± 138	4344 ± 247	312 ± 21	252 ± 30	564 ± 50	2292 ± 230	2240 ± 190	4532 ± 419	9440 ± 351
rmTBI (n=4)	1608 ± 151	1588 ± 164	3195 ± 310	168 ± 18	168 ± 31	335 ± 35	1543 ± 231	1578 ± 174	3120 ± 285	6650 ± 484

Supplementary table 3. Spatiotemporal hypothalamic SRPX2 expression after repeated mild lateral FPI (rmTBI). Table showing total number of SRPX2 immunoreactive neurons in the hypothalamic paraventricular (Pa), periventricular (Pe), and supraoptic (SO) nuclei of the rat brain after rmTBI. No statistically significant interhemispheric differences were identified. Note the reduction in number of SRPX2+ neurons in the Pa and hypothalamus after rmTBI. Data are expressed as mean ± SEM.

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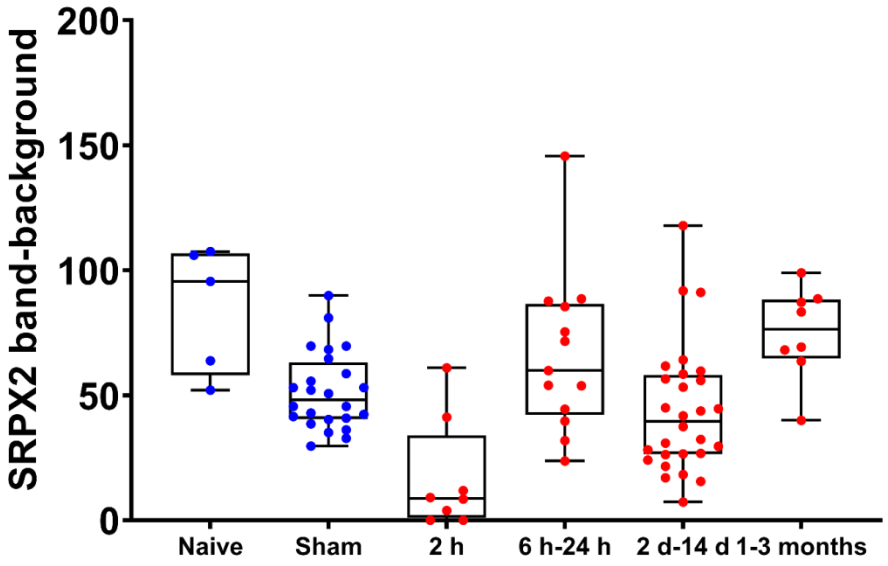

Supplementary figure 1. The plasma SRPX2 levels after severe TBI. The outcome of the quantification of SRPX2 band intensity with only subtraction of the background was similar to that of quantification after normalization for total protein and positive control. This indicated that normalization did not affect the overall interpretation of the plasma SRPX2 levels after TBI.

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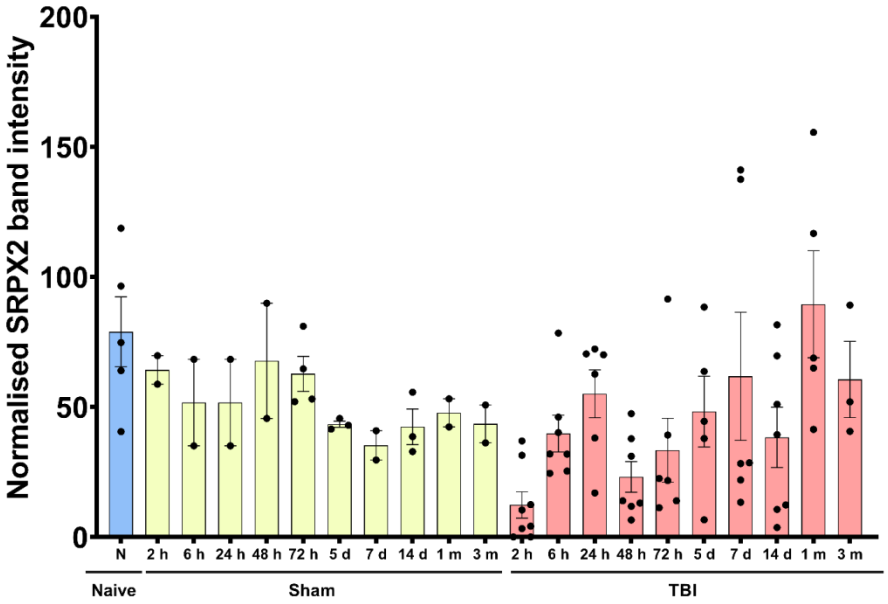

Supplementary figure 2. SRPX2 plasma levels after severe TBI. Quantification of Western blots at all time points after TBI. Due to variation in plasma SRPX2 levels within the TBI group, rats with high plasma SRPX2 (>1SD of mean of the control group [naïve + sham]) levels were compared with the ones with low plasma SRPX2 levels. There was no difference in the weight (371.7 ± 24 vs 367.8 ± 30), hit pressure (3.2 ± 0.06 vs 3.3 ± 0.07) and apnea time (49 ± 24 vs 29 ± 11) of the rats with higher plasma SRPX2 levels compared to low plasma SRPX2 levels ($p>0.05$).

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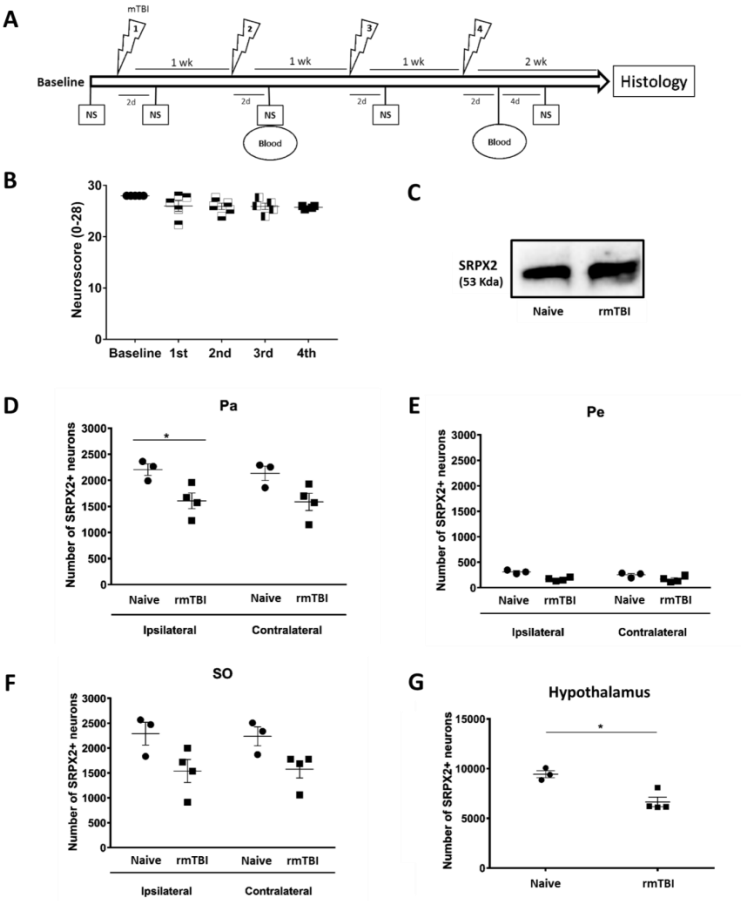

Supplementary figure 3. SRPX2 expression after repeated mild TBI (rmTBI). (A) Study design: induction of rmTBI, plasma sampling, and perfusion of brain for histology. Repeated mild TBI was induced by exposing the dura to 1.3 atm impact four times at 1-week intervals**. (B)** The composite neuroscore decreased ($p>0.05$) after the 1st mTBI but did not worsen with repeated injuries. **(C)** Plasma SRPX2 levels after the 4th rmTBI were comparable with plasma SRPX2 levels of naïve animals. **(D)** The number of SRPX2-ir neurons in the Pa decreased in rats with rmTBI compared with naïve rats. **(E-F)** The number of SRPX2-ir neurons in the Pe and SO in rats with rmTBI were comparable with that in naive rats. **(G)** Overall, the number of SRPX2-ir neurons in the hypothalamus decreased in rats with rmTBI as compared with naïve rats.