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ORIGINAL ARTICLE

A multi-staged neuropeptide response to traumatic brain injury

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Abstract

Purpose As the most abundant neuropeptides in Central Nervous System, Substance P and Neuropeptide Y are arguably involved in the response to brain trauma. This study aims to characterize a new concept of multi-staged neuropeptide response to TBI.

Methods This study assessed Substance P, Neuropeptide Y, S100B, standard inflammatory parameters and ionic disturbance in TBI victims, with and without intracranial lesions, and healthy controls. In the group with intracranial lesions, blood samples were drawn until 6 h after initial trauma, at 48 h and 7 days post-TBI.

Results An early increase in Substance P (mean 613.463 ± 49.055 SE 6 h post-TBI with brain contusions vs. 441.441 ± 22.572 SE pg/dL control group) is evident. Concerning TBI without intraparenchymatous lesions, an increase in substance P is also present (825.60 ± 23.690 SE pg/dL). Following an initial increase and subsequent fall in NPY levels (45.997 ± 4.96 SE 6 h post-TBI vs. 32.395 ± 4.056 SE 48 h post-TBI vs. 19.700 ± 1.462 SE pg/mL control group), a late increase in NPY is obvious (43.268 ± 6.260 SE pg/mL 7 day post-TBI). Post-traumatic hypomagnesemia (0.754 ± 0.015 SE 6 h post-TBI vs. 0.897 ± 0.021 SE mmol/L control group) and a peak in S100B (95.668 ± 14.102 SE 6 h post-TBI vs. 30.187 ± 3.347 SE pg/mL control group) are also present.

Conclusion A multi-staged neuropeptide response to TBI is obvious and represents a potential therapeutic strategy for the treatment of intraparenchymal lesions and cerebral edema following TBI.

Keywords Brain trauma · Neurogenic inflammation · Neuropeptide Y · Substance P

Introduction

Traumatic brain injury (TBI) is a major public health issue, with economic/social significance [1–3]. As a consequence of brain trauma, primary damage is followed by cellular/biochemical deregulation, neurometabolic disturbance, hippocampal synaptic disturbance and neuronal/astrocyte degeneration (CA1/CA3 layers, Dentate Gyrus) [4, 5], related to glutamatergic excitotoxicity [6–10]. Ensuing Blood–Brain Barrier (BBB) breakdown and neurogenic inflammation [11, 12] will then reinforce brain edema and early/late apoptosis [7]. Effective treatments are yet to be seen, despite recent developments in reliable biomarkers [13].

Increased post-traumatic levels of Substance P (SP) and perivascular immunoreactivity following TBI have been reported in the literature [14, 15]. Substance P, which is acting on NK1 tachykinin receptors (NK1-r) [16, 17], is partially responsible for post-TBI neurogenic inflammation, promoting vasogenic edema [18, 19], chemokine production and adhesion molecule expression [20]. Substance P is regulated by magnesium/NMDA-receptor signaling pathways [21]. Magnesium has been reported to be neuroprotective, regulating NMDA receptors and attenuating neuronal/astrocyte excitotoxicity. Other studies have shown a post-TBI decrease in intracellular and serum magnesium levels, influenced by SP [22].

Neuropeptide Y (NPY), the most abundant brain neuropeptide and major neurotransmitter [23], modulates the cytotoxic environment following stroke or epilepsy and it is suggested to support neuronal regeneration [24,

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25]. Neuropeptide Y influences neuroprotective pathways affecting glutamatergic hippocampal excitability (NPY Y2 receptors) and pro-neurogenic (NPY Y1 receptors) and pro-migratory activity (Dentate Gyrus, Sub-Ventricular Zone) [26–28]. Despite its ubiquitous influence, NPY´s response to TBI is relatively unknown.

S100 beta (S100B), an intracellular S100-group Ca^{2+}-binding protein [29], located primarily in astrocytes [30], is a useful TBI biomarker [31], displaying adequate sensitivity in detecting and assessing progression of brain lesions, as it is correlated with the amount of injured tissue [32, 33].

We hypothesize that TBI leads to a multi-staged neuropeptide response, as shown by an immediate response concerning SP, followed by compensatory NPY up-regulation. This study aimed at assessing neuropeptide response among different groups, including TBI with and without obvious brain injury, its temporal profile and relation to S100B and Magnesium levels, knowingly affected by TBI [3, 13].

Materials and methods

A prospective, single-center analysis of all consecutive patients with clinical diagnosis of TBI and and indication for head CT imaging was performed from January 2017 to July 2019 at the Hospitais Universitários de Coimbra (Coimbra's University Hospitals).

A thorough Informed Consent Form and the protocol for selection of patients, preservation of anonymity and handling of clinical data were approved by the Ethics Committee both in Centro Hospitalar e Universitário de Coimbra (Coimbra's Hospital and University Centre) and Coimbra's University.

Diagnosis of TBI was confirmed upon anamnesis, with confirmation of significant head trauma, and clinical examination. Timing of TBI with respect to clinical observation was confirmed by the patient (when appropriate), accompanying persons and/or medical teams involved in pre-hospital management. Indication for performing initial CT scan was in accordance with the Portuguese National Protocol in Traumatic Brain Injury—moderate to severe TBI, according to Glasgow Coma Scale (GCS) score; abnormal neurological examination; significant loss of consciousness; suspected fracture; known risk factors (> 65 year, alcoholism, drug abuse, epilepsy, coagulopathy, previous cranial surgery). CT scans (Siemens SOMATOM go-All®) were evaluated by an independent radiologist (from a group of six dedicated neuroradiologists) and classified as showing intraparenchymal lesions (contusions, hematomas) or not.

Exclusion criteria included: pediatric patients (17 years or less); patients > 80 years; active or recent infection; acute/chronic renal or gastrointestinal disease; inflammatory bowel disease; alcohol dependence; uncontrolled or recently diagnosed diabetes; recent vomiting/diarrhea; previous/present cranial or intracranial pathologies; concomitant cranial/intracranial traumatic findings (fractures; subdural/epidural hematomas; subarachnoid hemorrhage); simultaneous significant traumatic findings (thoracic, abdomen, limbs, spine or others); recent traumatic injuries; patients prescribed with diuretics, angiotensin converting-enzyme inhibitors, gentamicin, amphotericin and others, interfering with magnesium metabolism. Patients initially enrolled but who posteriorly (in initial 7 days post-injury) required any tipe of surgical procedure or developed an infectious or other significant medical condition were also excluded. All previously mentioned conditions or medications can potentially interfere with inflammation pathways, neuropeptides levels or ionic balance.

Patients were enrolled into the study upon meeting inclusion/exclusion criteria and assigned to their corresponding group following assessment of corresponding head CT scan findings.

Participants were divided into five groups, $n = 35$ per group (as $n > 30$ is usually considered a minimum for large samples regarding Central Limit Theorem): Control group (healthy volunteers)—*Group A*; TBI victims without parenchymal lesions (as shown on CT scans and despite significant head trauma), 6 h or less after trauma—*Group B*; TBI victims with visible parenchymal lesions (as shown on CT scans), 6 h or less after TBI—*Group C-6 h*; TBI victims with visible parenchymal lesions, 48 h after TBI—*Group C-48 h*; TBI victims with visible parenchymal lesions, 7 days after TBI—*Group C-7 day*.

A subset of patients, initially included in group C-6 h, underwent repeated samplings in all different timings (6 h, 48 h and 7 days) and is, therefore, included in all groups C.

Blood samples were collected via direct venipuncture, and immediately processed in Hospital's laboratories, as follows:

- Group A—elective blood sampling
- Group B—blood sampling until 6 h following TBI
- Group C-6 h—blood sampling until 6 h following TBI
- Group C-48 h—blood sampling 48 h following TBI
- Group C-7 day—blood sampling 7 days following TBI

Peripheral blood was collected to 8-mL heparin tubes (S-Monovette®), homogenized and transferred within a 5-min interval to a 15-mL Falcon tube containing aprotinin, preventing protein degradation (concentration 0.014 TIU/mL). After gently inverting, Falcon tubes were left to rest for 20 min. Falcon tubes were then centrifuged for 15 min at 1000*g* and 4 °C. Samples were stored in 200-µL aliquots at − 80 °C to prevent repetitive freeze/thaw cycles. Fifty µL of plasma was used in duplicates and absorbance was

determined (BioRad® model 600 plate reader). Average of duplicates readings was performed and a standard curve was generated using a four-parameter logistic curve-fit to determine plasma concentrations in pg/mL.

Determination of NPY, S100B and Substance P plasma levels was performed by Enzyme-Linked Immunosorbent Assay (ELISA) [(NPY and S100B kits Merck KGaA® (New Jersey, USA); SP kits R&D Systems® kit (Minnesota, USA)]. Dilutions for the ELISAs took place for SP (1:2, taken into consideration for final calculations). The following detection limits were used for the different assays: 16.8–43.8 pg/mL dynamic range for SP (according to the supplier); 2.7 pg/mL for S100B; 2 pg/mL for NPY.

Determination of Calcium, Magnesium, Sodium, Potassium, Chloride, C-Reactive Protein (CRP) and Osmolality was undertaken. Blood samples were processed on Architect analyzers (Abbot Diagnostics®): ionogram indirect potentiometry (Sodium, Potassium, Chloride); enzymatic assays (Magnesium); immunoturbidimetry and arsenazo III Calcium complexes assays.

Statistical analysis

All data were analyzed using IBM SPSS Statistics version 24.0 and are presented as mean ± standard error of the mean (SEM). For comparison of parametric results between multiple groups, one-way analysis of variance (ANOVA), followed by post-hoc Tukey's test (for equal sample sizes) or Tukey–Kramer test (for unequal sample sizes) was performed. Concerning the sub-group of patients who underwent repeated measures, a non-parametric Friedman Test was performed, according to the specific data requirements and non-gaussian distribution. A p value less than 0.05 ($p \leq 0.05$) was considered statistically significant.

Results

A total of 129 patients were included in the study, distributed in five distinct groups as outlined in "Materials and methods". For each group, 35 patients (or controls, concerning group A) were enrolled as an end-point—66.6% males in total (see Table 1 for group-specific findings). The average age was: 48.8 year ± 1.969 SE in group A; 61.4 year ± 3.206 SE in group B; 65.03 year ± 2.440 SE in group C-6 h; 65.06 year ± 2.477 SE in group C-48 h; 65.4 year ± 2.496 SE in group C-7 day (range, considering all groups, from 27 till 80 years). No specific findings with statistical significance were obvious concerning age or gender. Two patients (6%) initially enrolled in group C-6 h died in the first 48 h following TBI. From group C-6 h, 23 patients were carried over and included in subsequent groups C-48 h and C-7 day, forming a specific set of patients with consecutive sampling at 6 h, 48 h and 7 days following TBI (average age—63.8 year; 61% males, $n = 14$) (Table 2).

Missed samples and exclusion of obvious outliers explain differences in group sizes concerning obtained results.

At the moment of blood sampling, the number of patients who were admitted in Neurointensive Care Unit was as follows: group C-6 h—nine patients (25.7%); group C-48 h—ten patients (28.6%); and group C-7 day—nine patients (22.9%).

NPY

On one-way ANOVA test, there was a significant effect of TBI on NPY levels among different groups—[$F(4, 151) = 4.76$, $p = 0.0012$], post-hoc Tukey–Kramer method test (Fig. 1a). Significant increases in NPY levels are observed when comparing victims of TBI with brain contusions at 6 h and controls [group C-6 h (mean 45.997 ± 4.968 SE, $n = 32$) vs. group A (mean 19.702 ± 1.462 SE, $n = 31$)] and when comparing victims of TBI with and without brain contusions (at 6 h) [group C-6 h vs. group B (mean 29.567 ± 5.427 SE, $n = 29$)]. Neuropeptide Y is also significantly increased when comparing TBI with brain contusions at 6 h and 48 h post-TBI [group C-6 h vs. group C-48 h (mean 32.395 ± 4.056 SE, $n = 32$)]. A significant increase is again obvious if comparing victims of TBI with brain contusions at 7 days and controls [group C-7 day (mean 43.268 ± 6.260 SE, $n = 30$) vs. group A].

Considering the subset of patients with paired samples (repeated blood sampling in the same patient at 6 h, 48 h and 7 days post-TBI) ($n = 23$), a similar pattern in NPY levels is displayed (Fig. 1b)—significant rise within the first 6 h (mean 39.924 ± 6.487 SE), with NPY levels declining out to 48 h (mean 28.929 ± 4.867 SE) and increasing again until 7 days following TBI (mean 43.467 ± 8.072 SE). These differences in mean values did not reach statistical significance upon non-parametric Friedman Test for repeated measures: $\chi^2 - 5.826087$ ($\alpha - 0.05$; dF—2; χ^2 critical value – 5.99147).

In regard to NPY levels and its relation to initial GCS scores in group C-6 h, considerable differences were present (Fig. 2): mild TBI (GCS 14–15) —mean 40.114 ± 11.435 SE, $n = 13$; moderate TBI (GCS 9–13)—mean 29.460 ± 3.950 SE, $n = 10$; severe TBI (GCS 3–8)—mean 53.210 ± 11.910 SE, $n = 9$. Those differences did not reach statistical significance upon non-parametric three-groups comparison with Kruskal–Wallis test by ranks: $\chi^2 - 1.180461$ ($\alpha - 0.05$; dF—2; χ^2 critical value – 5.99147).

Substance P

On one-way ANOVA test, there was a significant effect of TBI on SP levels among different groups—[$F(4, 100) = 8.190$, $p < 0.001$], post-hoc Tukey–Kramer method

Table 1 General view on results concerning different groups

	Group A	Group B	Group C-6 h	Group C-48 h	Group C-7 day
N (initial)	35	35	35	35	35
Age (mean)	48.8 ± 1.969 SE	61.4 ± 3.206 SE	65.4 ± 2.440 SE	65.1 ± 2.477 SE	64.7 ± 2.496 SE
Age (max)	61	79	80	80	80
Age (min)	26	27	27	27	27
Male/female	21/14	23/12	24/11	20/15	21/14
GCS on admission (*n*)					
14–15	35	25	16	16	19
9–13		10	14	9	8
3–8			5	10	8
Deaths (7 days post-TBI)	–	–	2	–	–
NPY (pg/mL)	19.702 ± 1.462 SE (*n* = 31)	29.567 ± 5.427 SE (*n* = 29)	45.997 ± 4.968 SE (*n* = 32)	32.395 ± 4.056 SE (*n* = 32)	43.268 ± 6.260 SE (*n* = 30)
SP (pg/mL)	441.441 ± 22.572 SE (*n* = 31)	825.606 ± 23.690 SE (*n* = 30)	613.463 ± 49.055 SE (*n* = 26)	587.576 ± 48.363 SE (*n* = 26)	620.083 ± 46.743 SE (*n* = 27)
S100B (pg/mL)	30.187 ± 3.347 SE (*n* = 31)	42.303 ± 6.302 SE (*n* = 29)	95.668 ± 14.102 SE (*n* = 22)	71.778 ± 9.556 SE (*n* = 23)	58.860 ± 13.708 SE (*n* = 22)
Magnesium (mmol/L)	0.897 ± 0.021 SE (*n* = 35)	0.861 ± 0.039 SE (*n* = 29)	0.754 ± 0.015 SE (*n* = 33)	0.811 ± 0.019 SE (*n* = 34)	0.925 ± 0.039 SE (*n* = 34)
Calcium (mg/dL)	9.46 ± 0.063 SE (*n* = 35)	9.10 ± 0.102 SE (*n* = 35)	8.73 ± 0.149 SE (*n* = 35)	8.63 ± 0.098 SE (*n* = 35)	8.71 ± 0.135 SE (*n* = 35)
CRP (mg/L)	0.461 ± 0.244 SE (*n* = 35)	1.435 ± 0.518 SE (*n* = 35)	1.674 ± 0.469 SE (*n* = 35)	7.706 ± 1.106 SE (*n* = 35)	6.348 ± 1.244 SE (*n* = 35)
Sodium (mmol/L)	140.06 ± 0.415 SE (*n* = 31)	138.57 ± 0.570 SE (*n* = 31)	137.76 ± 0.682 SE (*n* = 31)	139.20 ± 0.718 SE (*n* = 31)	137.53 ± 0.816 SE (*n* = 31)
Potassium (mmol/L)	4.55 ± 0.101 SE (*n* = 33)	4.08 ± 0.073 SE (*n* = 34)	4.06 ± 0.066 SE (*n* = 28)	3.89 ± 0.082 SE (*n* = 31)	3.94 ± 0.105 SE (*n* = 31)
Chloride (mmol/L)	105.19 ± 0.338 SE (*n* = 35)	105.5 ± 0.630 SE (*n* = 35)	102.96 ± 0.552 SE (*n* = 35)	103.93 ± 0.828 SE (*n* = 35)	102.93 ± 0.854 SE (*n* = 35)
Osmolality (mmol/kg)	280.67 ± 0.983 SE (*n* = 35)	280.06 ± 1.168 SE (*n* = 35)	281.83 ± 1.465 SE (*n* = 35)	283.50 ± 2.204 SE (*n* = 35)	282.00 ± 1.629 SE (*n* = 34)

Table 2 Group C (patients with repeated samplings): patients demographics and GCS score

	Group C (repeated sampling)
n	23
Age (mean)	63.8 ± 2.596 SE
Age (max)	80
Age (min)	27
Male/female	14/9
GCS (*n*)	**GCS 14–15/9–13/3–8**
6 h	13/6/4
48 h	13/5/5
7 day	15/4/4

GCS score 14–15 - mild TBI; GCS score 9–13 - moderate TBI; GCS 3-8 - severe TBI

test (Fig. 3). A significant increase is obvious in group C-6 h (mean 613.463 ± 49.055 SE, *n* = 26) when compared to group A (controls) (mean 441.441 ± 22.572 SE, *n* = 31) and group B (mean 825.606 ± 23.690 SE, *n* = 30). Group C-6 h also displays higher SP levels when compared to group C-48 h (mean 587.576 ± 48.363 SE, *n* = 26). A significant increase is also present when comparing group C-7 day (mean 620.083 ± 46.743 SE, *n* = 27) and group A. Even in the absence of visible brain contusions, a significant increase in SP is demonstrated when comparing group B (TBI with no contusions) and group A.

S100B

On one-way ANOVA test, there was a significant effect of TBI on S100B levels among different groups—[$F(4, 95) = 4.959$, $p = 0.0011$], post-hoc Tukey–Kramer method test (Fig. 4). A significant increase takes place in the first 6 h post-TBI when compared to controls [group C-6 h (mean 95.668 ± 14.102 SE, *n* = 22) vs. group A (mean 30.187 ± 3.347 SE, *n* = 31)], followed by progression to baseline values in the next 7 days. S100B is also significantly increased in the presence of post-traumatic brain

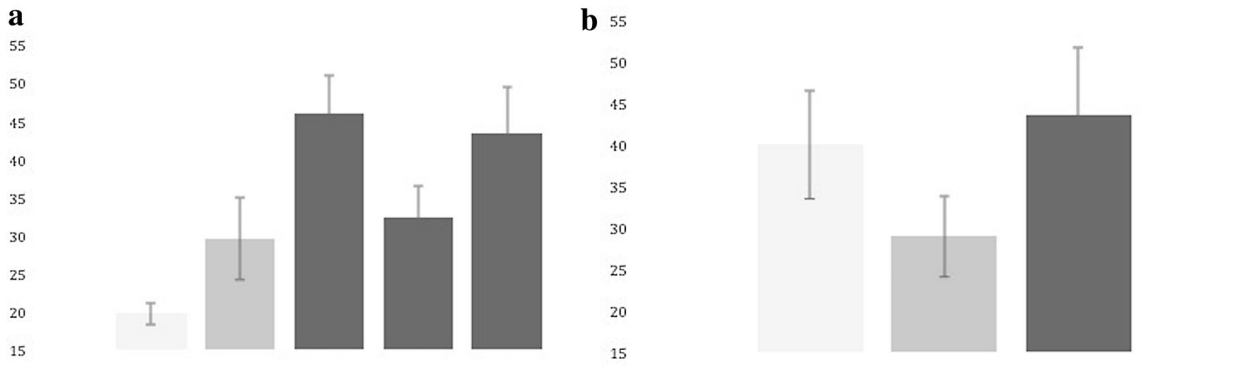

Fig. 1 **a** Response to TBI concerning NPY (pg/mL). Group A (Controls) – light grey; Group B (TBI) – grey; Group C (TBI with contusions; 6h, 48h and 7 days consecutively) – dark grey. Significant higher levels of NPY in group C-6h (mean 45.997 +/- 4.968 SE) and C-7d (mean 43.268 +/- 6.260 SE) when compared to group A (controls) (mean 19.702 +/- 1.462 SE), group B (TBI with no contusions) (mean 29.567 +/- 5.427 SE) and group C-48h (mean 32.395 +/- 4.056 SE). **b** Response to TBI concerning NPY, repeated sampling (pg/mL). 6h post-TBI – light grey; 48 hours post-TBI – grey; 7 days post-TBI – dark grey. Similar pattern when comparing distinct timings in the same patient: group C-6h - mean 39.924 +/- 6.487 SE vs. group C-48h - mean 28.929 +/- 4.867 SE vs. group C-7d - mean 43.467 +/- 8.072 SE. Stated differences did not reach statistical significance

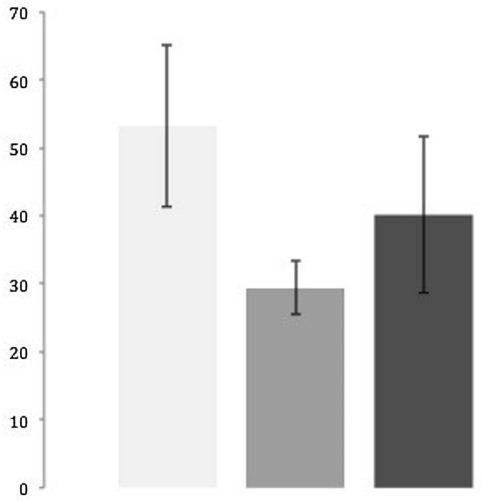

Fig. 2 Response to TBI concerning NPY, group C-6h, according to initial GCS scores (pg/mL). Mild TBI (light grey) (GCS 14-15), mean 53,210 +/- 11.910 SE; moderate TBI (grey) (GCS 9-13), mean 29.460 +/- 3.950 SE; severe TBI (dark grey) (GCS 3-8), mean 40.114 +/- 11.435 SE. Stated differences did not reach statistical significance

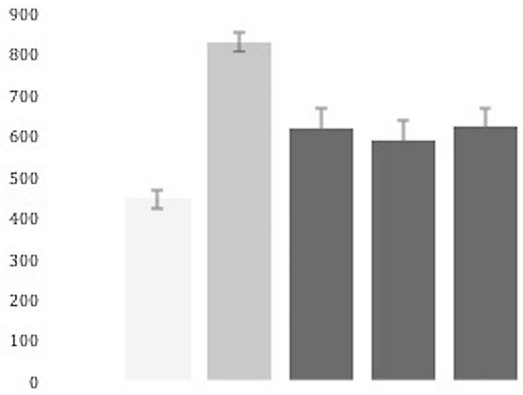

Fig. 3 Response to TBI concerning SP (pg/mL). Group A (Controls) – light grey; Group B (TBI) – grey; Group C (TBI with contusions; 6h, 48h and 7 days consecutively) – dark grey. Significant increase in group B (TBI with no contusions) (mean 825.606 +/- 23.690 SE) and group C-6h (mean 613.463 +/- 49.055 SE) when compared to group A (controls) (mean 441.441 +/- 22.572 SE)

contusions when compared to TBI with no contusions (both at 6 h) [group C-6 h vs. group B (mean 42.303 ± 6.302 SE, $n = 29$)].

Clinical laboratory tests

On one-way ANOVA test, there was a significant effect of TBI on Magnesium levels among different groups—[$F(4, 145) = 5.682$, $p < 0.001$], post-hoc Tukey–Kramer method test (Fig. 5). Significant hypomagnesemia is present when comparing victims of TBI with brain contusions at 6 h and controls [group C-6 h (mean 0.754 ± 0.015 SE, $n = 33$) vs. group A (mean 0.897 ± 0.021 SE, $n = 35$)] and when comparing different timings in all groups of TBI with brain contusions [group C-6 h vs. group C-48 h (mean 0.811 ± 0.019 SE, $n = 34$) vs. group C-7 day (mean 0.925 ± 0.039 SE, $n = 34$)], with progressive recovery of Mg levels following TBI. Mean levels of Mg are also lower when comparing victims of TBI with and without brain contusions (at 6 h) [group C-6 h vs. group B (mean 0.861 ± 0.039 SE, $n = 29$)].

Mean values of Sodium, Potassium and Osmolality were unremarkable in what concerns different groups.

On one-way ANOVA test, there was a significant effect of TBI on Calcium levels among different groups—[$F(4,$

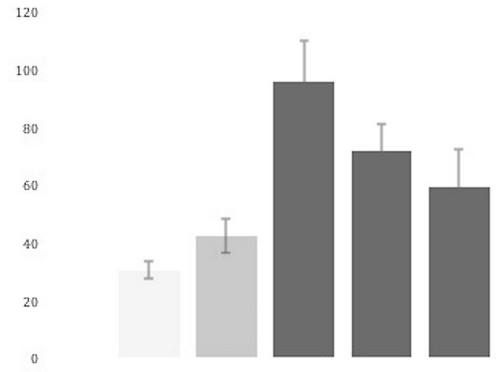

Fig. 4 Response to TBI concerning S100B (pg/mL). Group A (Controls) – light grey; Group B (TBI) – grey; Group C (TBI with contusions; 6h, 48h and 7 days consecutively) – dark grey. Significant increase in group C-6h (mean 95.668 +/- 14.102 SE) when compared to group A (controls) (mean 30.187 +/- 3.347 SE) and group B (TBI with no contusions) (mean 42.303 +/- 6.302 SE)

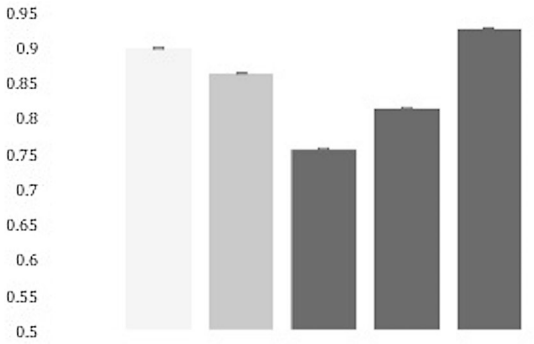

Fig. 5 Response to TBI concerning Magnesium (mmol/L). Group A (Controls) – light grey; Group B (TBI) – grey; Group C (TBI with contusions; 6h, 48h and 7 days consecutively) – dark grey. Significant hypomagnesemia in group C-6h (mean 0.754 +/- 0.015 SE) when compared to group A (controls) (mean 0.897 +/- 0.021 SE) and group B (TBI with no contusions) (mean 0.861 +/- 0.039 SE). Magnesium levels progressively recover to their baseline levels at 48h (group C-48h) (mean 0.811 +/- 0.019 SE) and 7 days (group C-7d) (mean 0.925 +/- 0.039 SE)

146) = 9.593, $p < 0.001$], post-hoc Tukey–Kramer method test (Fig. 6). A significant decrease is obvious when comparing controls and TBI with no brain contusions [group A (mean 9.46 ± 0.063 SE, $n = 35$) vs. group B (9.10 ± 0.102 SE, $n = 35$)], controls vs. TBI with brain contusions at 6 h [group A vs. group C-6 h (mean 8.73 ± 0.149 SE, $n = 35$)] and TBI with no brain contusions vs. TBI with brain contusions (both at 6 h) (group B vs. group C-6 h).

On one-way ANOVA test, there was a significant effect of TBI on CRP levels—[$F(4, 143) = 16.056$, $p < 0.001$], post-hoc Tukey–Kramer method test (Fig. 7). A significant increase takes place when comparing TBI with brain contusions at 48 h (mean 7.706 ± 1.106 SE, $n = 35$), TBI with

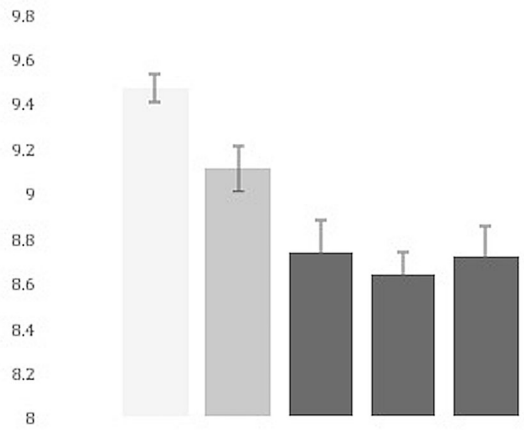

Fig. 6 Response to TBI concerning total serum Calcium (mg/dL). Group A (Controls) – light grey; Group B (TBI) – grey; Group C (TBI with contusions; 6h, 48h and 7 days consecutively) – dark grey. Significant hypocalcemia in group C-6h (mean 8.73 +/- 0.149 SE) when compared to group A (controls) (mean 9.46 +/- 0.063 SE) and group B (TBI with no contusions) (mean 9.10 +/- 0.102 SE)

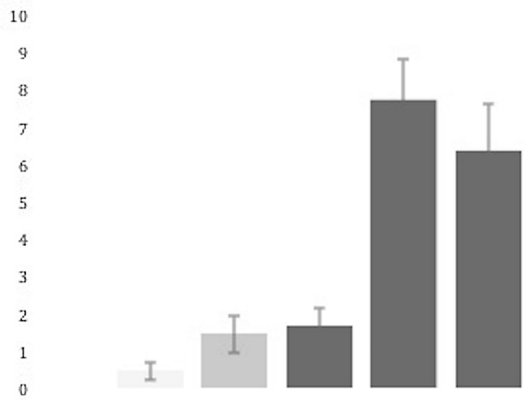

Fig. 7 Response to TBI concerning CRP (mg/L). Group A (Controls) – light grey; Group B (TBI) – grey; Group C (TBI with contusions; 6h, 48h and 7 days consecutively) – dark grey. Significant increase in CRP in relation to TBI and its different timings: group C-48h (mean 7.706 +/- 1.106 SE) vs. group C-6h (mean 1.674 +/- 0.469 SE) vs. group A (controls) (mean 0.461 +/- 0.244 SE)

brain contusions at 6 h (mean 1.674 ± 0.469 SE, $n = 35$) and controls (mean 0.461 ± 0.244 SE, $n = 35$) (Fig. 7).

Discussion

Despite growing interest in TBI's long-term consequences [34], several therapeutic protocols failed the test of facing modern evidence-based medicine [35]. Hypomagnesemia and SP's increment were tested as therapeutic targets [22], using specific antagonists (N-acetil-triptofan, canabinoid receptor-2 agonists) [35], with no clear benefits.

We hypothesized that TBI leads to a multi-staged neuropeptide response, with an immediate response concerning SP, followed by compensatory NPY up-regulation. This response is divided, based on previous literature and according to our working model, now confirmed by the previously depicted results, in three different moments (Fig. 8):

- A hyper acute response exacerbated by SP, as an inflammatory response in the first hours following TBI, promoting cerebral vasogenic edema and neuroinflammatory processes;
- –An acute response determined by excitotoxic phenomena, partially mediated by Substance P, and a peak in S100B levels as a sign of neuronal/glial disturbance, progressing cerebral edema and neurological impairment;
- Finally, a delayed response with significant increase in NPY levels (and possibly others) as reinforcement to neuroprotective pathways, attenuating excitotoxicity and neuroinflammatory phenomena, with ancillary progressive recovery in Mg levels.

As depicted in the "Results", evidence of an early and delayed neuropeptide response to TBI is, therefore, shown. These findings are in line with the perceived role for neuropeptides and neurogenic inflammation as key components of post-TBI inflammation, along with SP's role in aspects of classical inflammatory response (activation of microglia/astrocytes, leukocyte migration, degranulation of mast cells) [20]. A minor initial increase in NPY was followed by an expected and significant decrease at 48 h post-TBI (coinciding with the usual timing for peak clinical deterioration and known deleterious secondary injury on a celular level) [36–38].

To better assess the relevance and adequacy of our working model, namely regarding NPY, this research protocol contemplated the possibility of comparing distinct patients among different groups (control, TBI, TBI with brain lesions in different timings) and, on a specific sub-group, compare NPY´s levels in the same patient upon different timings. Similar trends in NPY´s fluctuating levels were confirmed in both contexts, although an inferior number of patients in the paired-samples (repeated measurements) analysis might have prevented it from reaching statistical significance. This, in our opinion, reinforces our conclusions, as the statistical relevance of comparing specific, separate groups of patients is supplemented by the notion of biological continuity, considering the same objective response in the subset of patients assessed upon different timings. Assessment of NPY levels according to initial GCS scores did not provide any additional information, a fact most likely related to significant clinical variability upon initial presentation and small-sized groups.

This staged neuropeptide response is also in agreement with the well-known timings in brain injury and its biomarkers, with well-described post-traumatic hypomagnesemia and S100B levels peaking in the first 48 h post-TBI and

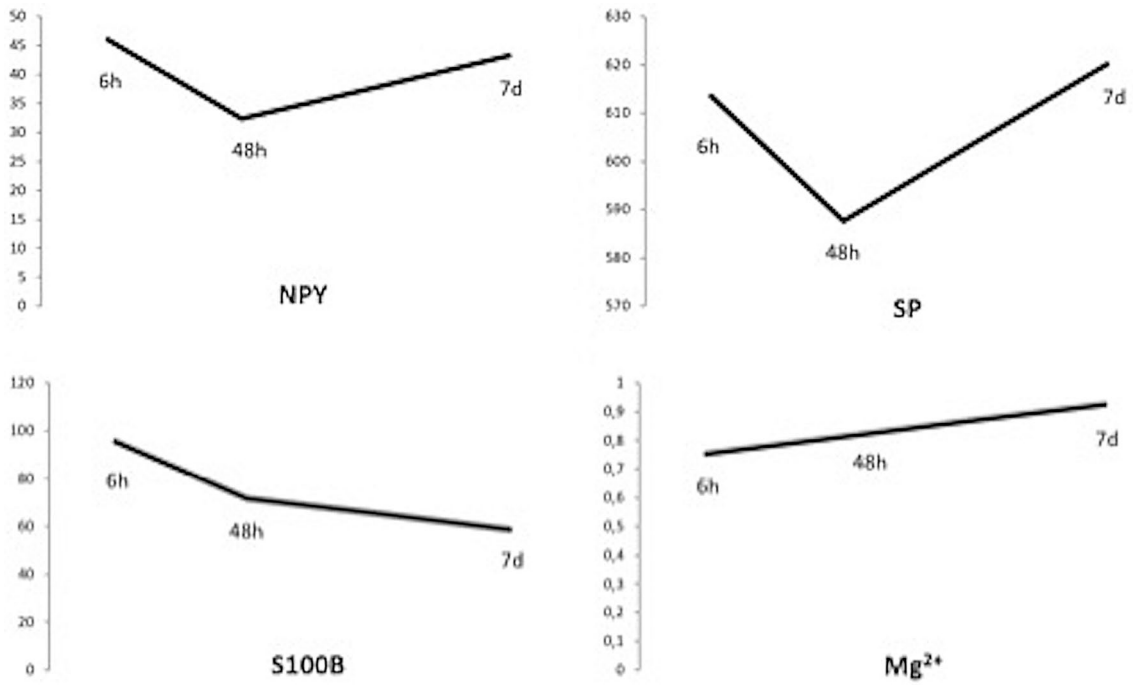

Fig. 8 Schematic representation of multi-staged response to TBI, with different timings for each element involved

subsequently normalizing (assuming stabilization of the clinical picture and no ongoing parenchymatous lesions) [39]. Other aspects of SP´s role in TBI still require better clarification, including the mechanisms behind its interference with BBB (some authors speculated on decreased endotelial expression of ZO-1 and claudin-5) [40].

Substance P levels in group B (TBI with no intracranial lesions) are increased when compared to controls (group A), an interesting finding that reinforces the relevance of TBI-related deleterious phenomena even in CT-negative patients. Rather counterintuitively, SP's levels are also further increased in group B when compared to group C, in which brain contusions are present. This fact, never reported before, can only be explained if considering a scenario on which neuroinflammation pathways upon TBI are surpassed by more relevant and disrupting events, including cell death and haemorrhages, in the context of direct brain injury with significant contusions. This would preclude the expected, but in this case impaired, neuroinflammation response, somewhat dependent on a more intact underlying brain structure. On the other hand, in groups C, an expected decrease at 48 h was followed by a increase in SP's levels at 7 days, unlike the typical pattern described in the literature [15]. This fact, never reported before, is of uncertain significance and should be better elucidated in future studies.

Other findings, although of undeniable clinical relevance, are somewhat expected and deemed not relevant in this context. An obvious post-traumatic hypocalcemia is present, most likely in relation to well-described iatrogenic hemodilutional mechanisms [41], as expected in patients subjected to Neurointensive Care protocols, sometimes with aggressive fluid management. Significantly high CRP levels were also present in TBI patients. Despite several authors and research teams exploring the possibility of using CRP as a biomarker for TBI [42], this possibility, beyond the scope of this work, does not seem valid or useful [43], given CRP's heterogeneity, clinical ubiquity and lack of specificity in complex patients as in polytrauma.

Although other neurotransmitters are likely to be involved in post-traumatic neurogenic inflammation, namely CGRP (which might potentiate SP's action) [20], this research project focused on SP and NPY, as the most ubiquitous and potent neuropeptides [44]. NPY takes part, via five different receptor types in several different biological processes and events [24], including feeding stimulus and obesity [45], stress modulation [46], homeostatic balance, sleep, olfaction perception [47], cyrcadian rythms and endocrinologic disturbances [48]. Recent reports mention a possible role for NPY in neuroprotective strategies regarding psychiatric diseases [49], Alzheimer's disease and Parkinson's disease [50, 51], along with possible roles in modulating neuropathic pain and behavioral changes in temporal lobe epilepsy [24]. Neuropeptide Y, as well as its selective NPY Y2 receptor agonist (NPY13-36), is known to act as an endogenous anticonvulsant by modulating glutamatergic hippocampal excitability [25], while displaying inhibitory action on cytotoxic cellular edema, by regulating KCl-evoked glutamate release [52]. Interestingly, NPY is co-localized with SP on GABAergic interneurons and A1 neurons in supra-optic nuclei (despite different ATP-stimulated actions on vasopressin and oxytocine release) [53], among other organs [54], with some authors suggesting that NPY might counterbalance SP's action, namely on nociceptive pathways and/or inflammation [55, 56].

Given all evidence pointing to a neuroprotective action of NPY in different contexts, it is plausible to consider a key role for NPY in brain's response to TBI, a field of knowledge where the gap is tremendous. Sporadic reports have shown elevated plasmatic and serum NPY levels in animal models of trauma and humans [57, 58]. Long-term changes in hippocampal NPY expression are related to occurrence of post-traumatic epilepsy in animal models of TBI [59]. Not surprisingly, brain NPY levels and function are reduced in the elderly [60], a significant group in TBI, reinforcing the importance of potentiating this response. As previously mentioned, NPY is known to act as an antagonist to SP's and other neuropeptides activity, inhibiting SP release with anti-hyperalgesic effect via Y1 receptor signalling in the dorsal horn [55]. Therefore, reinforcing NPY's response is a potential therapeutic strategy, possibly along with SP modulation [14], in Neurotrauma, considering previously mentioned NPY's pro-neurogenic, pro-migratory and neuroprotective properties [61]. Neuropeptide Y supplementation protocols (namely by intranasal delivery) are underway in phase II/phase III clinical trials concerning other clinical contexts [62, 63].

Some issues can be raised concerning this research protocol. First, CT scans were initially classified by six experienced radiologists. Although a possible source of bias, it is unlikely that significant errors might arise from a simple assessment on having or not brain contusions/hematomas, an objective and rather obvious finding in scans. The presence of intraparenchymal lesions was the only variable (concerning NPY/SP/S100B and the presence or not of brain contusions in TBI victims, as shown in this study)—the number and classification of those lesions was not considered for the purpose of this study. Variations in size and severity of those lesions should influence neuropeptide response, but our intention was to demonstrate an encompassing phenomenon, regardless of severity. Likewise, it was not intended to assess a possible relation between neuropeptide response, clinical status and outcome.

Reported variability of SP and NPY serum and plasma levels is another possible bias [64]. Distinct sample preparation, qualitative differences in reagents, distinct analytical methods and SP's plasma/serum free and bound states

could lead to wrong estimates [64, 65]. Besides difficulties in dealing with complex patients in a complex environment (lost or mixed samples, change of clinical status), there was significant difficulty in obtaining valid results concerning SP's and S100B, with missed samples and outliers explaining most discrepancies in groups size concerning results.

As previously mentioned, none of the measured proteins is brain specific. Given this, as the traumatic event is well identified and time specific, any significant changes in protein's levels should be a TBI's direct consequence and valid extrapolations can be made. On the other hand, determination of neuropeptide's plasma levels is technically difficult and, as some issues with outliers were present, a tendency to spurious results should be kept in mind.

The decision on different timings for blood sampling was based on clinical grounds and previous research. Post-traumatic vasogenic edema peaks around the 3rd day post-TBI, unlike immediate cytotoxic edema [66]. A 48-h time point seems suitable for an adequate mid-asessment; while the 7-day time point provides, in our opinion, a good notion on a longer term. Concerning blood sampling until 6 h post-TBI, one should not ignore rapid fluctuations in SP's levels—dividing data into sub-groups (e.g., 30 min vs. 2 h vs. 6 h) should provide additional and useful information. Given this, it was decided to keep the 6 h threshold, again based on clinical reasons: as in most terciary hospitals, most patients arrive the Emergency Department several hours after initial trauma; as all doctors know, patient and family reports are seldom reliable; it would be relatively impractical to repeatedly collect blood samples in a trauma patient in such a narrow time frame. Significantly, our data display significant changes at 6 h post-TBI, assuming an eventual underrepresentation. The decision on not taking additional blood samples in group B (at 48 h and 7 days following TBI) was based on clinical reasons, considering the nature of this study, the early discharges (as no traumatic findings were present) and the unnecessary blood sampling.

Conclusion

A multi-staged neuropeptide response to TBI is demonstrated. Future experimental studies (including animal models of trauma) should further characterize this response and eventually assess its potential as a therapeutic target, aiming at maximum functional recovery.

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Availability of data Supporting data can be accessed upon request.

Compliance with ethical standards

Conflict of interest None.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Institutional Research Committee (Centro Hospitalar e Universitário de Coimbra) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

Consent for publication All authors have consented for the publication of this work.

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