Species specific ultrastructural properties of excitatory neocortical synapses and synaptic changes in postpartum depression

PhD Thesis

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Introduction

The cerebral cortex is one of the most complex cellular structures. The human cerebral cortex consists of 10^{10} neurons and equal number of glial cells, which accounts for 90% of all the cellular elements. The number of synaptic junctions connecting neurons ranges from 10^{12} to 10^{14} depending on gender, age, and health.

The main task of our brains is to process, store and respond to an ever-changing and vast amount of information from external and internal environments. These tasks are put into effect by the variety and plasticity of the neurons and their synaptic connections. From 5000 up to 60 000 synaptic junctions can contact a single cortical neuron. These synaptic contacts were first revealed by Held and Auerbach with an aniline blue staining procedure. Later, Sherrington coined the term "synapse" and defined as the point of information transfer between neurons. The widely accepted quantal model of the synaptic transmission was formulated by Castillo and Katz and since then the anatomical background of the synaptic transmission has became clear. The neurotransmitter is stored in vesicles of the axon terminals of the presynaptic cell. During synaptic transmission, these vesicles release their neurotransmitter content into the synaptic cleft, exerting its effect upon the postsynaptic cell. The vesicles anchored to the presynaptic active zone (docked vesicles) are waiting to be released by a presynaptic action potential arriving to the axon terminal. The quantum nature of synaptic transmission is caused by the action of these vesicle neurotransmitter packets.

Neurons of the cerebral cortex can be divided into two major categories. The majority of the cells (80%) are the excitatory pyramidal cells, excreting glutamate as their neurotransmitter. The other class of cortical neurons are the diverse group of local inhibitory GABAergic internurons. Pyramidal cells of the cerebral cortex excite local inhibitory interneurons via their glutamatergic axon terminals. Over the past decades several studies were performed to elucidate the workings of these connections in rodents, the most commonly used animal model. The excitatory postsynaptic potentials evoked by these connections in the rodent cortex have an average amplitude of 1 to 4 mV, which is hardly enough to drive the postsynaptic interneuron to fire action potentials. As opposed to this, these specific connections are far more efficient in the human cerebral cortex: a single action potential in the presynaptic pyramidal cell is sufficient to depolarize the surrounding fast spiking axo-axonic and basket cells above their firing threshold. Single action potentials of human neurons are

sufficient to recruit Hebbian-like neuronal assemblies that are proposed to participate in cognitive processes.

The hippocampus, just like the cerebral cortex, has a laminar organization. The excitatory glutamatergic principal cells of the hippocampus fall in two morphological categories. The granule cells are found in the stratum granulosum of the dentate gyrus (DG) with their densely spined short dendrites. The other morphological group consists of the pyramidal cells of the Cornu ammonis (CA) with their somas located in the stratum pyramidale. Pyramidal and granule cells, the principal hippocampal neurons, are organized sequentially into a 'trisynaptic loop', which is the main neuronal circuitry of the hippocampus. Asymmetric spine synapses within the CA1 stratum radiatum, the CA3 stratum lucidum and radiatum, as well as in the dentate gyrus stratum moleculare represent primary connections of the trisynaptic loop.

Compromised hippocampal function in major depression is correlated with reduced hippocampal volume, suppressed adult hippocampal neurogenesis, and loss of spine synapses along the trisynaptic loop, and these structural impairments are all prevented/reversed by effective antidepressant treatment. The central role of the hippocampus in major depression is further supported by the observation that local infusion of brain-derived neurotrophic factor or neurotrophin-3 into the dentate gyrus reproduces the effects of conventional antidepressants. Although recent studies have demonstrated hippocampal structural modifications that are related to maternal stress and mood disorders, direct electron microscopic evidence of synaptic remodeling along the trisynaptic circuit in PPD is presently not available.

Similar to major depression, epidemiological studies show that exposure to stress is a significant risk factor of PPD. Like in the case of humans, female rats exposed to peripartum stress develop depressive and anxiety-like behaviors, as well as reduced maternal care in the postpartum period. It has been suggested, in line with the synaptogenic hypothesis of depression, that peripartum stress elicits extensive neuroplasticity in the maternal brain, which potentially contributes to the development of postpartum affective illness. Recent studies are beginning to demonstrate that the maternal nervous system is indeed remodeled by stress both in humans and in animal models. Nevertheless, more research is needed to better understand the extent to which neuroplasticity is implicated in stress-related maternal disorders.

In order to model the stress and the hormone withdrawal aspects of PPD, we used the hormone withdrawal model of Suda and colleagues combined with inescapable footshock stress applied during the 'postpartum' period. Our primary goal was to provide evidence that exposure to 'postpartum' stress leads to loss of asymmetric spine synapses along the

hippocampal trisynaptic circuit. We also hypothesized that the association between loss of hippocampal synapses and depressive behavior is maintained in the simulated postpartum environment.

Aims:

The first part of my PhD thesis deals with the ultrastructural differences between rodent and human cortical synapses using light- and electron microscopic techniques. Our specific question was the following:

1. What is the anatomical difference between human and rodent local excitatory synapses arriving onto cortical basket cells that makes these synapses in the human cortex more efficient than their rodent counterparts?

The second part of my PhD thesis deals with the anatomical background ot postpartum depression. Our specific questions were the following:

2. What is the effect of postpartum stress in a postpartum model?
3. Does the postpartum stress cause changes in the number of hippocampal spine synapses?

Materials and methods:

1. Species specific ultrastrastructural properties of excitatory neocortical synapses

Brain slice preparation

All procedures were performed according to the Declaration of Helsinki with the approval of the University of Szeged Ethical Committee. Human slices were derived from material that had to be removed to gain access for the surgical treatment of deep-brain tumors from the left and right frontal, temporal, and parietal regions with written informed consent of

female (n = 15, aged 53 ± 13 years) and male (n = 11, aged 43 ± 24 years) patients prior to surgery. After surgical removing blocks of tissue were immersed immediately in ice-cold artificial cerebrospinal fluid. Coronal slices (350 mm) were prepared from the somatosensory cortex of male Wistar rats (P18–P28). Slices were cut perpendicular to cortical layers at a thickness of 350 mm with a vibrating blade microtome (Microm HM 650 V) and were incubated at 30**°C** for 1 hr in the same solution.

Electrophysiology

Somatic whole-cell recordings were obtained at 36**°C** from simultaneously recorded doublets of pyramidal cells and fast spiking interneurons visualized by infrared differential interference contrast videomicroscopy in the layer II/III of the cerebral cortex. The cells were filled intracellularly with biocytin for post hoc anatomical analysis.

Light microscopy

After electrophysiological recordings slices were fixed in a fixative containing 4% paraformaldehyde, 15% picric acid and 1.25% glutaraldehyde in 0.1 M phosphate buffer (PB; pH = 7.4) at 4˚C for at least 12 hr. After several washes in 0.1 M PB, slices were cryoprotected in 10% then 20% sucrose solution in 0.1 M PB. Slices were frozen in liquid nitrogen then thawed in PB, embedded in 10% gelatin and further sectioned into slices of 60 mm in thickness. Sections were incubated in a solution of conjugated avidin-biotin horseradish peroxidase (ABC; 1:100; Vector Labs) in Tris-buffered saline

(TBS, pH = 7.4) at 4˚C overnight. The enzyme reaction was revealed by 3’3-diaminobenzidine tetrahydrochloride (0.05%) as chromogen and 0.01% H2O2 as oxidant. Sections were post fixed with 1% OsO4 in 0.1 M PB. After several washes in distilled water, sections were stained in 1% uranyl acetate, dehydrated in ascending series of ethanol. Sections were infiltrated with epoxy resin (Durcupan) overnight and embedded on glass slices. Three dimensional light microscopic reconstructions were carried out using Neurolucida system with 100x objective.

Electron microscopy

Visualization of biocytin and correlated light and electron microscopy (LM and EM) were performed as described previously. Dendritic segments (distance from the soma ~50 mm in rat and ~150 mm in human) of biocytin filled basket cells (identified based on distinctive electrophysiological properties and LM investigation of the axonal arbor) were re- embedded and re-sectioned at 20 nm thickness. Digital images of serial EM sections were taken at 64 000 magnification with a FEI/Philips CM10 electron microscope equipped with a MegaView G2 camera. Dendrites and the presynaptic boutons were 3D-reconstructed using the Reconstruct software. The areas of active zones were measured at perpendicularly cut synapses, where the rigid apposition of the pre- and postsynaptic membranes was clearly visible, and the docked vesicles were identified as described previously (Holderith et al., 2012). Active zones were identified by the parallel rigid membrane appositions where the synaptic cleft widened (because the PSD is masked by the dark DAB precipitate). Potential inhibitory synapses with flattened vesicles were discarded from the analysis. Bouton volumes of a subpopulation of boutons were measured from 20 nm serial reconstructions where the series contained the whole terminal (n = 9 human boutons, n = 8 rat boutons). We calculated the volume of the same subset of boutons from the area of the largest cross-section (assuming that boutons are spherical) and found a tight correlation (r = 0.9019, Spearman correlation) with the measured volume. Based on this correlation, we calculated the volume from the largest cross-section of those terminals where the AZ was measured, but the series did not contain the whole terminal.

EM tomography

200 nm thick sections were cut and collected onto copper slot grids. Fiducial markers (Protein-A conjugated to 10 nm gold particles, Cytodiagnostics - Absource Diagnostics GmbH, Munich, Germany) for tomographic reconstruction were introduced at both sides of the grids as described by Imig et al. (Imig et al., 2014). Single-axis tilt series of perpendicularly oriented synapses were acquired in FEI Tecnai G2 Spirit BioTWIN transmission EM operating at 120 kV and equipped with an Eagle 4K HS digital camera (FEI Europe Nanoport, Eindhoven, The Netherlands). Tilt series were

recorded between ± 65˚ (with 2˚ increments between ± 45˚ and with 1˚ increments between 45–65˚) at a magnification of 30,000 using FEI Xplore3D for automated tilt series

acquisition. Tomographic volumes were reconstructed using the IMOD package (Imig et al., 2014; Kremer et al., 1996) and exported as z-stacks for analysis. Active zone area and vesicle distance from the presynaptic membrane were measured with Reconstruct software. A vesicle was considered to be docked if the outer part of the lipid bilayer was in direct contact with the inner part of the presynaptic membrane bilayer. A vesicle was considered to be pre-docked if the distance between these lipid bilayers did not exceed 5 nm, and the membrane proximal vesicles are defined as the docked and pre-docked vesicles. For vesicle pool measurements, synaptic vesicles within 100 nm of the AZ were quantified from tomographic subvolumes and normalized to the AZ area according to Imig et al,(2014) in human and rat samples (n = 15 each).

2. Synaptic changes in postpartum depression

Animal groups

Adult female Sprague–Dawley rats were kept under standard laboratory conditions (n = 76, 200–250 g, Charles-River Laboratories, Vác, Hungary). Rats were treated and cared for according to National Institutes of Health standards. To prevent interference from endogenous ovarian hormones, all animals were ovariectomized on day-1 using a ketamine-based anesthetic (25 mg/mL ketamine, 1.2 mg/mL xylazine, 0.03 mg/mL acepromazine in saline, 3 mL/kg, i.m.). During the same surgical session, 21-day continuous release pellets (Innovative Research of America, Sarasota, Florida) containing 0.5-mg 17β-estradiol and 50-mg progesterone were implanted subcutaneously in the scapular region to simulate pregnancy. These pellets produce a dose of 23.8 μg/day for 17β-estradiol and 2.4 mg/day for progesterone, which approximate the average daily doses applied in the hormone simulated pregnancy model of Galea and colleagues. In contrast to the model of Galea and colleagues, which administers progesterone only until ‘pregnancy’ day 16, we withdrew 17β-estradiol and progesterone simultaneously on day-21 by removing the hormone pellets. Day-22 to day-28 was then considered as the early ‘postpartum’ period. As a result, our model provided pregnancy levels of hormones that were suitable for the rat system and a simulated postpartum period that was more relevant to human conditions by reproducing the simultaneous withdrawal of 17β-estradiol and progesterone.

Behavioral test

We applied an inescapable stress paradigm with a testing apparatus that consists of a commercially available shuttle avoidance box (Med Associates, St. Albans, Vermont) that is divided into two equal compartments with a central barrier. The barrier is equipped with a computer-operated guillotine door to provide passage between compartments. Animals subjected to inescapable stress received 60 scrambled footshocks with 0.85-mA intensity, being administered via wire grid flooring in a closed shuttle box compartment. The footshock protocol was entirely automated and conducted by a computer-run algorithm to provide fully randomized stress exposure with 15-s average shock duration and 45-s average intershock interval. Sham-stressed controls underwent the same footshock protocol, but the shock generator was switched off during the entire procedure. In order to verify the relationship between the number of hippocampal spine synapses and depressive symptoms, we applied active escape testing that is also routinely used in our laboratories to assess depressive behavior in rodent models. 30 trials of *escapable* footshock were administered with 0.65-mA intensity in the same shuttle avoidance box as described above. In contrast to inescapable stress, the guillotine door in the central barrier was automatically opened at the beginning of each footshock to provide the animal with opportunity to escape by passing between shuttle box compartments.The testing protocol was entirely automated and conducted by a computer-run algorithm to administer trials in a randomized manner with 35-s maximum trial/footshock duration and 60-s average intertrial interval. The initial five fixed ratio one trials, when one shuttle crossing terminated the footshock, were followed by 25 fixed ratio two trials, when two shuttle crossings were required to terminate the footshock. As main measures of depressive behavior, escape latencies and escape failures were registered, representing the time to escape footshock and the number of trials during which escape requirements were not met, respectively. In case of each escape failure, escape latency was set to 35 s.

Histology and electron microscopy

All animals scheduled for electron microscopic analysis were sacrificed under deep ketamine-based anesthesia (see above) by transcardial perfusion of phosphate-buffered saline followed by a fixative containing 4% paraformaldehyde and 0.1% glutaraldehyde dissolved in 0.1 M phosphate buffer (pH 7.4). Brains were dissected out and postfixed overnight in the same fixative without glutaraldehyde.

Each hippocampus was cut into 100-µm thick serial sections in the coronal plane using a vibratome. At each counting site, serial ultrathin sections were prepared, and pairs of digitized electron micrographs were taken from the same area of neighboring ultrathin sections at a final magnification of 11,000× (physical disector). Asymmetric spine synapses were then counted according to the rules of disector technique, utilizing an unbiased counting frame superimposed onto the electron micrographs. All counts taken from the same sampling area were summed and divided by the analyzed volume to calculate an average spine synapse density (synapse/μm^3 = sum of synapse counts/number of disectors per sampling area/single disector volume of 5.94 μm^3). Spine synapse density was finally multiplied by sampling area volume to arrive at the total number of spine synapses in that particular sampling area.

Results and discussion

1. Species specific ultrastrastructural properties of excitatory neocortical synapses

Excitatory postsynaptic currents, recorded from interneurons in response to single presynaptic action potentials showed a wide range of amplitudes in both species, with a significantly larger mean amplitude in human INs (human: 258.8 ± 272.8 pA; rat: 75.8 ± 58.7 pA; $p<0.001$, MWU-test). A potential explanation for the differences in EPSC amplitudes between human and rat is a larger number of synaptic contacts connecting the PC axons to the postsynaptic IN dendrites in the human cortex. To assess the number of putative synaptic contacts, we searched for close appositions of presynaptic axon terminals and postsynaptic dendrites under light microscopy (LM). The presynaptic axon from the parent PC soma to the target IN dendrites were reconstructed in nine human and fifteen rat cell pairs. Comparison of the number of LM detected synaptic contacts between human (3.3 ± 1.5; range: 1–6) and rat (2.9 ± 1.5, range: 1–6) revealed no significant difference ($p = 0.35$, MW U-test).

Finally we assessed potential ultrastructural correlates of the above described differences in the EPSC amplitudes in individual axon terminals. We performed three-dimensional reconstructions of presynaptic axon terminals from 20 nm thick serial EM sections. Biocytin-filled, post hoc recovered human (n = 3) and rat (n = 3) interneurons were chosen and axon terminals that established asymmetrical synaptic contacts on selected dendrites were fully reconstructed. First we compared the size of presynaptic AZs and found

that both human and rat active zones (AZs) have variable sizes (human: 0.02–0.26 mm2; rat: 0.02–0.08 mm2), but the human AZs were on average twice as large. Each bouton contained only a single AZ in both species. When we counted docked vesicles in these AZs using the 20 nm thin serial section approach we found a significantly larger number in human (4.2 ± 2.2 / AZ) compared to rats (1.3 ± 0.8 / AZ; $p<0.001$, MW U-test), resulting in a twice as large docked vesicle density in human. However, as the precise identification of docked vesicles is challenging in conventional EM even if very thin (20 nm) sections are used, we repeated these measurements using EM tomography; the best currently known method for this application. Analysis of EM tomograms in 3 human (n = 33 synapses) and 3 rat (n = 31 synapses) samples revealed a similar two-fold difference in the mean docked vesicle density. These results confirmed that reconstructions from very thin serial EM sections also provide a reliable measure of the number of docked vesicles. The large variability in the AZ area and number of docked vesicle prompted us to investigate their relationship and found a positive correlation between these parameters in both species. Furthermore, we found significantly larger bouton volumes in humans (0.14 ± 0.09 mm3, n = 20) compared to rats (0.05 ± 0.02 mm3, n = 17; $p<0.01$, MW U-test).

Paired recordings, post hoc anatomical reconstructions and high resolution EM analysis revealed that presynaptic AZs contain on average 4 docked vesicles in human, but only 1 in rats. Our data allowed us to provide the first estimate of the size of Katz's functional release site (del Castillo and Katz, 1954) of cortical synapses; approximately 0.012 mm2 AZ membrane area in human and 0.025 mm2 in rats. Thus, the space that harbors a functional release site or a docking site seems to be substantially smaller in human AZs. This raises an interesting question: why the molecular machinery necessary for the assembly of a functional release site needs less space in human? Answering this question requires quantitative proteomic analysis of these AZs at nanometer resolutions. Furthermore, it will be also interesting to see whether this species-specific difference is valid for all central synapses or it is a unique feature of the cortical microcircuit.

2. Synaptic changes in postpartum depression

Control group (Veh): Exposure of Veh rats to inescapable stress caused a decline in the number of spine synapses across all hippocampal areas ($P < 0.04$, Tukey–Kramer test). In the active escape task, the Veh group achieved a mean escape latency of 22.89 ± 1.87 s and made, on average, 13.04 ± 2.37 escape failures.

Hormone-withdrawn ‘postpartum’ group (PpD): Exposure to and subsequent withdrawal of pregnancy levels of female reproductive hormones did not change synapse numbers in sham-stressed PpD animals ($P > 0.2$, Tukey–Kramer test). In the PpD group again, inescapable stress caused a decrease in the number of spine synapses across all hippocampal areas ($P < 0.01$, Tukey–Kramer test). As a result, post-stress synapse numbers in the PpD group were not significantly different from those in untreated controls ($P > 0.25$, Tukey–Kramer test). PpD females executed the active escape task with a mean escape latency of 22.16 ± 1.97 s and with an average escape failure of 12.75 ± 1.99. These escape measures were not significantly different from those of untreated controls ($P = 0.989$ for escape latency, Tukey–Kramer test, and $P = 0.482$ for escape failures, Mann–Whitney U test). Escape performances of untreated controls and PpD females were practically identical.

Simulated proestrus group (ProE): Exposure of sham-stressed ProE rats to proestrus concentrations of gonadal steroids elicited a rise in synapse levels across all hippocampal areas ($P < 0.02$, Tukey–Kramer test). In the ProE group as well, inescapable stress caused a decline in the number of spine synapses across all hippocampal areas ($P < 0.01$, Tukey–Kramer test). As a result, post-stress synapse numbers in CA1sr and DGsm of the ProE group were not significantly different from those in untreated controls ($P = 0.054$, and DGsm of ProE/IS vs. DGsm of Veh/IS, $P = 0.063$, Tukey–Kramer test). By contrast, post-stress synapse numbers remained higher in CA3sl/sr of the ProE group ($P < 0.001$, Tukey–Kramer test). In the active escape task, the ProE group achieved a mean escape latency of 18.71 ± 1.21 s and made, on average, 12 ± 1.16 escape failures. This escape performance did not differ significantly from that of untreated controls ($P = 0.367$ for escape latency, Tukey–Kramer test, and $P = 0.430$ for escape failures, Mann–Whitney U test).

Hormone-treated ‘postpartum’ group (Horm): Continued exposure to pregnancy levels of gonadal steroids during the ‘postpartum’ period did not increase synapse levels in sham-stressed Horm rats ($P > 0.6$, Tukey–Kramer test). It even caused a moderate decline in CA1sr ($P < 0.02$, Tukey–Kramer test), reducing synapse numbers to post-stress levels ($P = 0.647$, Tukey–Kramer test). Contrary to the other three groups, inescapable stress elicited no change in the number of hippocampal spine synapses in the Horm group ($P > 0.15$, Tukey–Kramer test). As a result, post-stress synapse numbers in the Horm group remained higher in CA3sl/sr and DGsm relative to those in untreated controls ($P < 0.01$, Tukey–Kramer test). Horm rats executed the active escape task with a mean escape latency of 13.56 ± 1.39 s and with an average escape failure of 3.71 ± 0.83. These escape measures were better than those of all other groups ($P < 0.01$ for escape latency, Tukey–Kramer test, and $P < 0.002$ for escape

failures, Mann–Whitney *U* test), except that the escape latency of the Horm group did not differ significantly from that of simulated proestrus animals ($P = 0.196$, Tukey–Kramer test)

The present study demonstrates that during a simulated postpartum period, hormone-withdrawn 'postpartum' females respond to inescapable stress with loss of hippocampal spine synapses that is equivalent with what is observed in untreated controls, a female rat model of major depression. These data support our hypothesis that exposure to 'postpartum' stress leads to hippocampal spine synapse loss. We also show that sustaining pregnancy levels of ovarian hormones during 'postpartum' stress exposure is sufficient to overcome the synaptolytic effect, as inescapable stress caused no change in the number of hippocampal spine synapses in hormone-treated 'postpartum' rats. It has to be noted, however, that synaptoprotection is not fully confirmed in CA1sr, as pre-stress synapse numbers in this area of hormone-treated 'postpartum' rats were already at post-stress levels of untreated controls. It appears that this synaptoprotective effect is mediated, at least partly, by a muted stress response as measured by serum corticosterone levels. On the other hand, maintaining proestrus levels of gonadal steroids during 'postpartum' stress exposure is not capable of exerting similar synaptoprotective effects.

Our active escape experiment reveals that same post-stress levels of hippocampal spine synapses are coupled with practically identical escape performance (PpD group vs. Veh group). Higher post-stress numbers of hippocampal spine synapses, on the other hand, are associated with improved escape performance (Horm group vs. Veh group). Moreover, slightly higher post-stress synapse levels in simulated proestrus animals are related to slightly, but not significantly, better escape performance (ProE group vs. Veh group). These findings support our hypothesis that the relationship between synapse loss and depressive behavior, that we have first described in a female rat model of major depression, is retained in a simulated postpartum environment.

We can thus conclude that two important aspects of our synaptogenic hypothesis of depression, i.e., the synaptolytic effect of stress and the association between synapse loss and depressive behavior, appear to be valid in an animal model of PPD. As a result, the synaptic and the escape impairments of our PPD model (PpD group) are equivalent with those of an established animal model of major depression (Veh group).

The thesis is based on the following publications

Molnár, G., Rózsa, M., **Baka, J**., Holderith, N., Barzó, P., Nusser, Z., & Tamás, G. (2016) Human pyramidal to interneuron synapses are mediated by multi-vesicular release and multiple docked vesicles. *Elife*, **5**, 1–12.

IF: 8,303

Baka, J., Csakvari, E., Huzian, O., Dobos, N., Siklos, L., Leranth, C., MacLusky, N.J., Duman, R.S., & Hajszan, T. (2017) Stress induces equivalent remodeling of hippocampal spine synapses in a simulated postpartum environment and in a female rat model of major depression. *Neuroscience*, **343**, 384–397.

IF: 3,231

Other publications

Rózsa, M., **Baka, J**., Bordé, S., Rózsa, B., Katona, G., & Tamás, G. (2017) Unitary GABAergic volume transmission from individual interneurons to astrocytes in the cerebral cortex. *Brain Struct. Funct.*, **222**, 651–659.

IF: 5,811

Faragó, N., Kocsis, Á.K., Braskó, C., Lovas, S., Rózsa, M., **Baka, J**., Kovács, B., Mikite, K., Szemenyei, V., Molnár, G., Ozsvár, A., Oláh, G., Piszár, I., Zvara, Á., Patócs, A., Barzó, P., Puskás, L.G., & Tamás, G. (2016) Human neuronal changes in brain edema and increased intracranial pressure. *Acta Neuropathol. Commun.*, **4**, 78.

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Cumulative impact factor: 17,345