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Article

Ectopic Expression of Cation Chloride Cotransporters KCC2 in Blood Exosomes, used as a Biomarker of Functional Rehabilitation

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Abstract: Background. Traumatic brain injury (TBI) is the major cause of disabilities in the industrialized countries. Cognitive decline appears in the chronic phase of the pathology consecutively to cellular and molecular processes. Here we described the use of KCC2, a neuronal-specific potassium-chloride transporters as potent biomarker to predict cognitive dysfunctions after TBI; **Methods.** Using neuronal and total exosomes collection from blood serum in control and TBI subjects we were able to anticipate the decline of cognitive performance; **Results.** After TBI, we observed a significative and persistant loss of KCC2 expression in the blood exosomes that is correlated to changes in network activity and cellular processes such as secondary neurogenesis. Also we correlated this KCC2 loss in expression to the appearance of the cognitive decline observed in mice and more particularly we correlate the KCC2 loss of expression to the appearance of the depressive-like behavior; **Conclusion.** According to our protocol, we were able to confirm our previous findings in agreement with the potential therapeutic effect of bumetanide in the prevention of the post traumatic depression after TBI, by restoring the KCC2 expression thus preventing the massive neuronal death of interneurons and the secondary neurogenesis effect observed in such model.

Keywords: psychiatric disease; brain injury; chloride transporter

1. Introduction

TBIs are the main causes of handicap all over the world with an high frequency in developed countries (Bondi et al., 2015; Meyer et al., 2008), according to the World Health Organization (Meyer et al., 2008). Brain trauma are classified according multiple factors such as altered neurological functions, brain areas and genetic variations. Taken together this leads to highly individualized and specific injuries. Long-term consequences of brain traumas include post-traumatic epilepsies, which severity and occurrence depend on trauma severity (Bragin et al., 2016; Kelly et al., 2015), cognitive dysfunctions and depressive-like behaviors are also associated (Peeters et al., 2015; Perry et al., 2015). Alleviating the consequences of TBI is crucial both socially and economically (Kessler et al., 2009) as the most of the people have difficulties to recover a proper life with a return to professional life and as the associated cost for the society is highly elevated. Posttraumatic depression (PTD) appears at high prevalence after TBI and presents with depressed mood, loss of interest or pleasure, disturbed sleep or appetite and poor concentration (Darren W Roddy et al., 2018). PTD often comes with decreased cognitive performances and could become chronic leading to substantial impairments in an individual's ability to take care of everyday responsibilities (WHO, 2008). Recovery and early diagnosis after TBI are of main interest, and among the available therapies, very few exhibit massive and permanent

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effect. Kinetic of syndrome appearance is crucial for the settling up of disease, first there is asymptomatic phase called latent phase during which TBI induce tissue damage and cellular death leading to inflammatory/immunological responses in the brain and blood–brain barrier (BBB) breakdown, this phase also promote secondary brain damage (Agoston and Kamnaksh, 2018; Vigil et al., 2019) and network rearrangement leading to epilepsy (Epsztein et al., 2005; Kourdougli et al., 2017; Sloviter, 2008). Then the chronic phase takes place, it is accompanied of cognitive decline (Santhakumar et al., 2001) and modification of cellular processes such as secondary neurogenesis of the hippocampus (Carli et al., 2020; Ibrahim et al., 2016). This sequence of events is found both in rodent (Goubert et al., 2019) and in human (Sankar and Mazarati, 2010). It has been proposed that changes in GABAergic neurotransmission is largely involved in such cascade of events. First parvalbumin-containing interneurons have been shown to be susceptible to death after both epilepsy and brain injury leading to increased hyperexcitability of neuronal network (Avramescu et al., 2009; Chandrasekar et al., 2018). Interestingly, all brain regions are facing the same trajectory from hippocampus to cortical layers and deep nuclei (Avramescu et al., 2009; Hsieh et al., 2016). In much neurological and psychiatric pathology, GABAergic pathways deregulation is implicated through chloride transport impairment. It has been demonstrated that down regulation of the neuronal-specific chloride and potassium extruder, KCC2, together with the up regulation of the chloride importer NKCC1 are common factors observed in that cases (Medina et al., 2014). Such effects on GABAergic transmission have been also reported in different models of neurodevelopmental diseases (Ben-Ari, 2017). Change in chloride transporters expression leads to facilitated depolarization and may emulate an excitatory action of GABA that may affect the generation of physiological relevant oscillations of brain networks (Kahle et al., 2013; Lüscher and Fuchs, 2015; Rivera et al., 1999). This depolarizing effect of GABA transmission has been observed thereby in neurodevelopmental disorders such as autism spectrum disorders (Tyzio et al., 2014), in stroke (Jaenisch et al., 2010) and epilepsies (Kelley et al., 2016; Pallud et al., 2014). The coupling between major depressive disorders (MDDs) and GABAergic neurotransmission has been proposed in a genetic mice model of GABA(B) receptor depletion (Mombereau et al., 2005) and in publications demonstrating the potential antidepressant effect of GABA(A) transmission blockage (Rudolph and Knoflach, 2011) in different brain regions such as the hippocampus (Boldrini et al., 2013) and the mesolimbic system (Kandratavicius et al., 2014). The resulting pathologies are associated with GABA receptors chanelopaties in granule cell layer (GCL) based on alterations of GABAA receptors composition (Drexel et al., 2014), but also to mossy fibers sprouting of the hippocampus dentate gyrus (Hunt et al., 2013) or metabolic hyperexcitability (Jakkamsetti et al., 2019). Some others hypotheses are found in the literature to explain the appearance of PTD. Previous results showed that chloride homeostasis is also involved in cell survival (Pellegrino et al., 2011), regulation of neurotrophin signaling (Shulga et al., 2008) and inflammation (Pin-Barre et al., 2017) both *in vitro* (Shulga et al., 2012) and *in vivo* (Goubert et al., 2019; Kourdougli et al., 2017). Alteration of secondary neurogenesis is an hallmark marker of depression (Carli et al., 2020; Tunc-Ozcan et al., 2019), the contribution of parvalbumin-containing interneurons to the migration of new-born cells and their intrinsic connectivity has been also reported (Bao et al., 2017; Ngwenya and Danzer, 2018) as well as their role as anti-depressant effector (Hu et al., 2017; Lauber et al., 2016). Altogether those events are in favor of a progressive appearance of the PTD that might be early determined using appropriate markers. In that context, biomarkers are of primary importance in the early diagnosis of pathologies. Biomarkers are defined as a biological characteristic related to normal or pathological activity and can be measured in different biological fluids or organs. They can be used to detect a disease, predict its severity or assess the effectiveness of a treatment (Patel, 2014). Several studies have shown the role of circulating exosomes as a new source of biomarkers for pathologies such as cancer or metabolic syndromes (Liu and Cao, 2016). Exosomes are nano-vesicles ranging from 30 to 100 nanometer diameter, secreted by different cells and detected in all biological fluids (Caby et al., 2005). They may transport nucleic acids, proteins and lipids specific to their cell of

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origin (Beach et al., 2014). They were originally described as a mean for reticulocytes to renew their membrane proteins, more particularly to eliminate proteins and RNAs that are no longer useful for the cell (Vlassov et al., 2012), more recently it has been shown their role in communication and intercellular material transfer, cell cycle, regulation of the immune response, antigen presentation and pathogen propagation (Sharma et al., 2019). They were proposed as structures involved in the metastasis processes (Liu and Cao, 2016) and as circulating vesicles able to carry materials involved in the change of the microenvironment (Kumar and Deep, 2020). Other publications have highlighted their biomarker role, in traumatic brain injury (Ko et al., 2018; Patel, 2014), depression (Brites and Fernandes, 2015), in the cell to cell communication and secondary neurogenesis (Sharma et al., 2019). The involvement of exosomes in immunity and intercellular communication suggests a huge potential of these vesicles as diagnostic and/or prognostic biomarkers in human pathology and especially as an early diagnosis tool for brain injury and their consequences as the cellular and molecular processes always precede the appearance of cognitive decline. Interestingly, it's possible to distinguish neuronal-specific ones from total exosomes based on the proteins that composed their membrane (Bagheri Hashkavayi et al., 2020; Bahrini et al., 2015; Beach et al., 2014). One big issue in the use of a biomarker is the correct identification of their origin. Taking into account the difficulty to make the brain cells express different transgene *in vivo*, we recently developed an original approach able to substitute to viral injection (Di Scala et al., 2018) and allowing us to discard in between neuronal expression from central nervous system to peripheral expression. This methods will allows us to tag the brain proteins in order to follow them.

2. Materials and Methods

Stereotaxic procedure. 10-weeks old C57bl6-J mice were housed in an enriched environment at INMED animal facility, maintained in a 12h light / 12h dark cycle environment with controlled temperature (23 ± 2 °C), food and water were given ad libitum. The stereotaxic procedure was performed using aseptic technique. Briefly, 30 min before surgery, buprenorphine (0.03 mg/kg) was given intra-peritoneally (i.p) then mice were anesthetized using 4% isoflurane vaporized by air together with additional 0.3% oxygen enrichment. Surgical anesthesia was maintained using 2% of isoflurane with 0.3% oxygen while mice were positioned in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). Body temperature was maintained at 37 ± 2 °C with a heating pad (Harvard Apparatus). Stereotaxic coordinates were chosen to allow injection above CA1 (reference to bregma, antero-posterior axis: -1, laterality: -1.2, verticality: -1.5). The skull was drilled while leaving the dura intact. Using a micro injector (micropump 4, WPI) 1.5 microliters were injected at 100 nL/min speed using NanoFil® D needle (WPI). After the injection the needle was left in place for an additional 15 minutes to prevent back-flow of liquid. Altogether the total duration of the surgery did not overpass 40 min. The skin was sutured and animals placed in the post-operative room of the facility. Brains were then either perfused and fixed for immunochemistry or freshly harvested for Western blot analysis 1 week after the injection.

Controlled-cortical impact model (CCI). 10-weeks old C57bl6-J males were housed individually in an enriched environment and maintained in a 12 hours circadian cycle under controlled temperature (23 ± 2°C) and with permanent access to food and water. The controlled cortical impact (CCI) procedure was performed using aseptic technique. buprenorphine (0.03 mg/kg) was injected intra-peritoneally (i.p) 30 min before surgery. The mice were then anesthetized using 4% isoflurane vaporized by air together with additional 0.3% oxygen enrichment. During surgical procedure, anesthesia was maintained with 2 to 2.5 % of isoflurane/0.3% oxygen in air. The mice were positioned in a stereotaxic frame (David Kopf Instruments). Body temperature was monitored throughout the procedure using a rectal probe and maintained at 37 ± 2°C with a heating pad (Harvard Apparatus). An unilateral craniotomy was performed over the right parietal cortex within the boundaries of the bregma and lambda while leaving the dura intact, using a high-

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speed drill. CCI was performed using a Leica impactor using the following parameters; tip diameter 3mm, 6 m/sec speed, 1.5 mm depth and 200 msec duration. The impact perpendicularly compressed the curvature of the sensori-motor cortex. Animals were allowed to recover on the heating pad before their transfer to the post-surgical room. Before the start of the experiments, animals were randomly assigned to subgroups, namely sham-vehicle +/- brainFectin, sham-bumetanide +/- brainFectin, CCI-vehicle +/- brainFectin and CCI-bumetanide +/- brainFectin. Bumetanide injections were performed i.p twice daily during for a one week period at 2 mg/kg concentration.

Blood Collection. Every animal was daily checked after CCI procedure for weight loss and general aspect according to the protocol validated by our local committee (Apafis #2797). For blood collection, we used the mandibular vein to collect blood from sham and TBI animals and a heart puncture when larger volume was needed, from transfected animals. After applying a local sanitizer, to the cheek, the mandibular vein is pierced perpendicularly with a lancet. Blood drops are collected in a blood tube while respecting the maximum volume. A small pressure is then applied to stop bleeding. We used appropriate Lancet (blood lancet Nahita FM024/60425012), and the maximum blood volume allowed to be collected was respected (Table 1). For heart puncture, after deep anesthesia using isoflurane 4% the animal is put in the supine position. After applying a local sanitizer to visualize the depression of the xyphoid appendix, we used a 25-gauge needle. Insert the needle gently into the xyphoid hollow at an angle of 30 ° to 45° to the top. Withdraw the larger volume of blood, if blood does not come out slightly alter the location of the needle.

Table 1. Maximum blood volume to be collected according to the puncture sites.

Location of blood collection	Approximate volume
Mandibular vein	100-200 µL
Cardiac puncture	0.5 to 1.0 mL

Total exosomes collection. Exosomes were isolated from 100 microliters of blood serum, after addition of 5µl protease and phosphatase inhibitor (pierce protease and phosphatase inhibitor Mini tablets, EDTA-FREE, invitrogen A32961). Total exosomes were first isolated from blood serum, using "total exosome isolation reagent/from serum" (Thermofisher scientific, Invitrogen 4478360) according to manufacturer's recommendation. After collection, exosomes were kept at 2°C to 8°C for up to 1 week, or at -20°C for longer storage. Neuronal fraction was enriched by immunoprecipitation using L1CAM antibody (eBio5G3 (5G3), thermo fisher scientific, #14-1719-82) but probe with CD63 to not interfere with the KCC2 staining.

Immunohistochemistry. Mice were deeply anaesthetized with i.p injection of ketamine/xylazine then transcardially perfused with cold phosphate buffer saline. (PBS 0.01 M) then 3% paraformaldehyde solution (AntigenFix,Diapath). Brains were post-fixed overnight in 3% paraformaldehyde at 4 °C and then coronally sliced with a Leica VT1200S Vibratome. 60 µm-thickness sections were permeabilized and blocked in PBS with 0.3% Triton X-100 and 5% normal goat serum (NGS) for 1 h at room temperature, stained with primary antibodies diluted in PBS with 5% NGS and 0.1% Triton X-100 at 4 °C overnight using anti-Dsred (Takara Bio Clontech, living colors polyclonal antibody, 632496), anti-NeuN (Merck Millipore; MAB377) and anti-Gad67 (Merck Millipore, MAB5406). After washing using PBS, slices were incubated with the corresponding Alexa Fluor 488 and 555-conjugated secondary antibodies diluted in PBS (1/500, Thermo Fisher Scientific, Invitrogen A11001) for 2 h at room temperature and finally counterstained for 1 min with Hoechst 33258 (10 µg/mL in PBS, Sigma-Aldrich, 94403). Sections were mounted onto Superfrost Plus glass slides in Fluoromount G mounting medium. For each section, serial images were taken using Fluorescence microscope equipped with an apotome module with 20× or 40× objectives.

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Protein extraction and Western blot. Animals were killed by decapitation after deep isoflurane anesthesia. Hippocampi were quickly dissected out, flash-frozen in liquid nitrogen and stored at −80 °C. Brain tissues were homogenized in RIPA buffer (50 mM Tris-HCl pH 8; 150 mM NaCl; SDS 0.1%; Deoxycholic Acid 0.5%; 1% Triton X-100) supplemented with Protease/Phosphatase Inhibitor Tablet (Thermofisher). Proteins were run on polyacrylamide gel (Bolt 4-12% Bis Tris plus, Invitrogen by Thermo Fisher Scientific) and transferred to a nitrocellulose membrane (GE Healthcare Life Science). After blocking in Tris-buffered saline/ 0.1% tween/ 5% bovine serum albumin (BSA), membranes were exposed overnight at 4 °C to primary antibodies diluted in blocking solution (Tris-buffered saline/ 0.1% tween/ 2.5% BSA). anti-Dsred (Clontech, Living Color Ds-Red Polyclonal Antibody, 632496), anti-KCC2 (home-made antibody, (Ludwig et al., 2003)), and Tubuline-β3 (Biolegend, 802001). Horse radish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Agilent Dako) were used as secondary antibodies, diluted in 5% bovine Albumin at room temperature for 2 h, finally bands were detected using Super Signal West Pico (Thermo Fisher Scientific, #34080) using the image analysis software G box (Syngene). Quantifications were performed using Gel Plot Analyzer plugin on Image J.

Drug delivery. 20 mM stock solution of bumetanide (Sigma-aldrich, B3023) was prepared by dissolving 36.4 mg of powder in 1 ml absolute ethanol. Mixing 40µL of stock solution with 4 mL of 1X PBS makes the injected solution. 26.7 µL per animal body weight gram of bumetanide is then injected intra-peritonealy (2 mg/kg), twice daily (9 am and 5 pm). Vehicle solution consists in the same procedure just lacking the bumetanide powder to respect volume and diluent.

Plasmid construct. The KCC2-mCherry construct was created by insertion of an ubiquitin promoter instead of CMV promoter in pmCherry vector (Clontech) for better expression of the transgene in *in vivo* conditions. Vector and transgene were sequenced before cloning and the full plasmid was also sequenced.

Statistical analysis. All mean values are given with the standard deviation error (SD). Normality was tested for each distribution and was set to 5%. Two-tailed Student's, Mann–Whitney test or one-way ANOVA were used accordingly using Prism software (GraphPad Software, Inc., La Jolla, CA, United States). Box plot report the median, the interquartile range and the total range data and represent as following: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

3. Results

3.1. Does KCC2 detectable in the circulating blood?

Our first investigation was about the possibility to detect KCC2 in the blood serum. A previous study has detected it in cerebro-spinal fluid (Duarte et al., 2013), so it appears crucial first to check for its presence in that biological fluid. After taking 0,2 mL of blood from the mandibular vein of mice, we proceed to serum isolation by centrifugation, 2.000g for 30 minutes at 4°C. After such procedure, we run western blot in denaturing condition on the clarified serum. As shown in Fig.1A we clearly see full length KCC2, 140kDa band from blood serum in all considered samples (n=30). Using our homemade antibody that recognizes the N-terminus part of KCC2 (Ludwig et al., 2003) we cannot clearly consider degraded form of KCC2, but gels bands profile, analyze by red Ponceau, did not show any difference with brain tissue samples Fig.1B (data not shown). As intriguing as it is, we wanted to know if the KCC2 molecule was secreted in the blood or if it was depending on other conditions.

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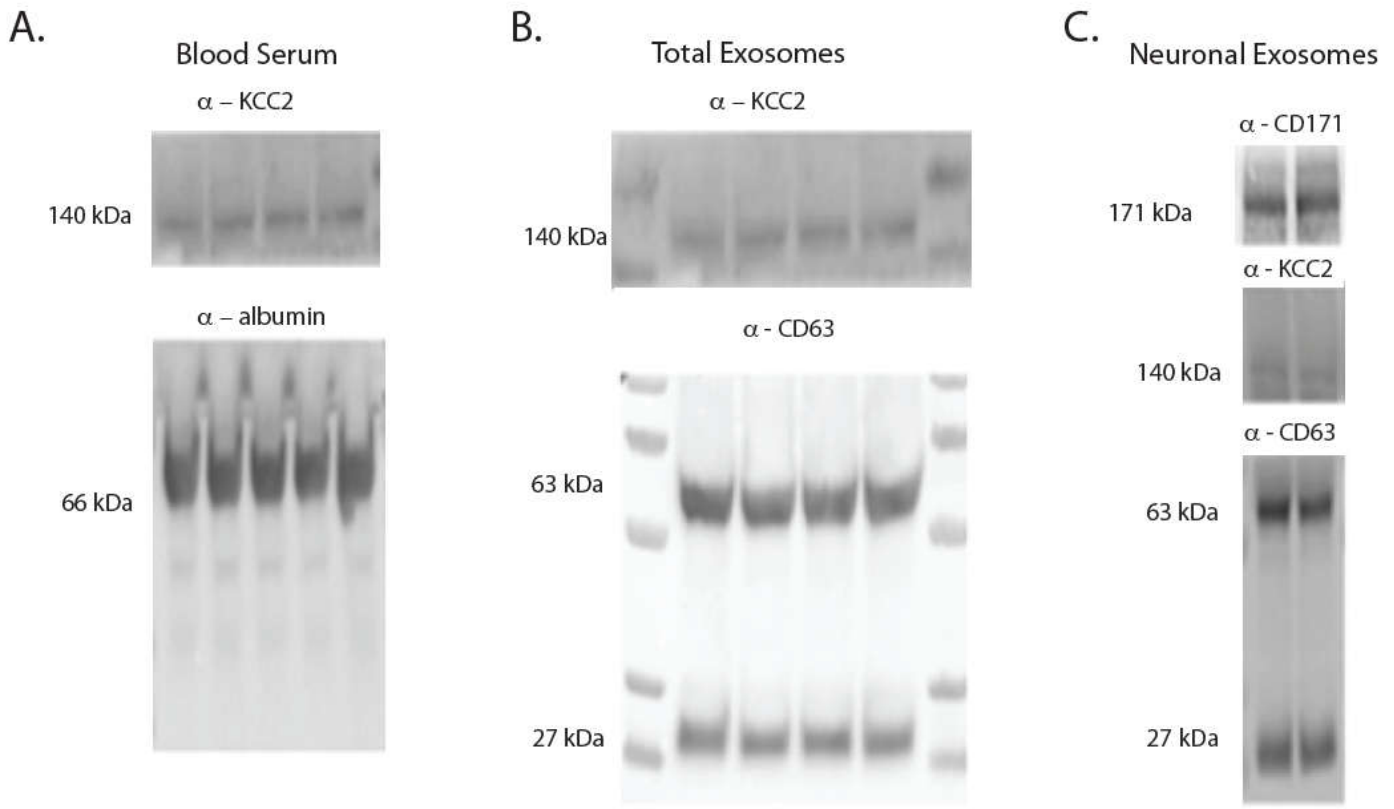

Figure 1. Identification of KCC2. A. in Blood serum (140kDa). **B.** KCC2 in brain tissue (hippocampus) shows the same band detected in serum which confirm that the band belong to KCC2. **C-D:** KCC2 is identified in total exosomes as well as neuronal derived exosomes that were purified from serum and following by confirmation of their neuronal origin by detecting the exosomal marker CD171. **E-F:** identification of mcherry-KCC2-tag in serum and in total and neuronal exosomes upshifted by 35kDa (KCC2 140kDa + mcherry 35kDa).

3.2. Do KCC2-containing exosomes are found in blood serum?

In the next step we wanted to check for KCC2 presence in the circulating exosomes coming either from neuronal cells or total cells, as these vesicles are known to diffuse over long distances. Using specific extraction kit we purified total exosomes from peripheral serum by immunoprecipitation. Isolation of total exosomes was confirmed by detecting the pan-exosomal marker, CD63 (Bagheri Hashkavayi et al., 2020), in each sample by Western blot (Fig.1B). The figure displays both glycosylated form (63kDa) and non-glycosylated forms of CD63 proteins (27kDa) with a predominance of the glycosylated form at 63kd. To go further on that and to confirm the neuronal origin of KCC2 we then perform purification of neuronal exosomes from total exosomes samples again by immunoprecipitation. We confirmed so far the neuronal-enriched KCC2 origin by western blotting using the neuronal exosome marker, L1CAM (CD171) (Pulliam et al., 2019) (Fig.1C).

3.3. Do KCC2- containing blood exosomes have neuronal origin?

KCC2 is almost exclusively expressed in the central nervous system (Blaesse et al., 2009), so it is intriguing to find full-length form of KCC2 in the circulating blood. To confirm the CNS origin of the molecule, we decided to genetically modified brain neuronal cells by *in vivo* transfection using BrainFectin® agent (Di Scala et al., 2018). Using stereotaxic approach, we injected DNA encoding a mCherry-tagged KCC2 in the C-terminal part of the molecule. After one week, brains were harvested and we proceed to co-immunoprecipitation. We already showed using our system, a potent and sustained cells

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modification of CNS cells using BrainFectin® (Di Scala et al., 2018). By performing post hoc analysis one week after injection we showed the expression of transgene by immunohistochemistry, using a specific antibody against the mCherry tag, in the injection site, CA1 (Fig. 2A-a) and also in the dentate gyrus (DG) (Fig. 2A-b). The high magnification in CA1 region of the hippocampus (Fig.2B) confirmed the robust expression of the mCherry-tagged KCC2. We confirmed the neuronal expression of the transgene in the principal neurons using neuronal marker NeuN (Fig.2C) and also in interneurons using Gad67 antibody (Fig.2D). We also confirmed at the protein level the presence of the mCherry-tagged in the ipsilateral hippocampal extract, this region was chosen because the hippocampus is the region targeted by the injection (1,76±0,20, n=5 vs 1.0±0,15, n=5, p=0,03. Fig.2E-F). As we have already shown by immunohistochemistry, there is no diffusion of the transgene in the contralateral side (0,83±0,17, n=5 vs 1.00±0,13, n=5, p=0,84 Fig.2G-H).

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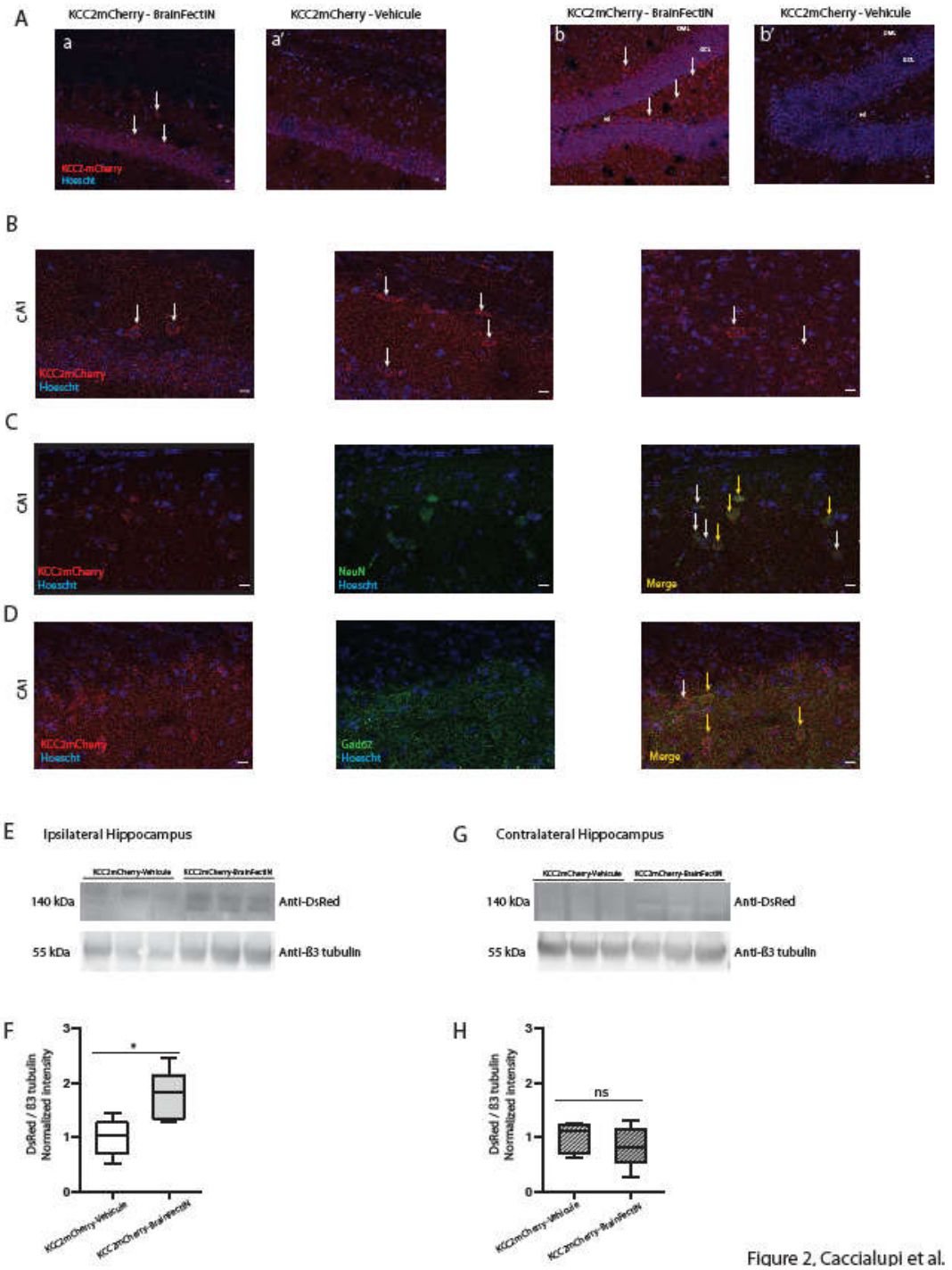

Figure 2. Figure 2. mCherry tagged KCC2 expression in the hippocampus one week after KCC2-mCherry- BrainFectiN injection. A. Expression of KCC2-mCherry in the ipsilateral CA1 area (a) and the ipsilateral dentate gyrus (b) (white arrows). Sham mice were injected with KCC2-mCherry construct and physiologic serum instead of the BrainFectiN and shown no positive cells in CA1 area (a') and dentate gyrus area (b'). Hi : Hilus, GCL : Granule Cell Layer, OML: Outer Molecular Layer. Scale bar = 10µM. Magnification 20x. **B.** Magnification of the injection site, CA1 area, showing the expression of the mCherry tagged KCC2. Scale bar=10µM. Magnification 40x. **C-D.** Representation of NeuN (C) and Gad67 (D) staining of KCC-mCherry positive cells (yellow arrows), white arrows indicate the noncolocalisation. Scale bar = 10µM. Magnification 40x. **E.** Representative western blots of hippocampi extracted from KCC2-mCherry-BrainFectiN injected mice (n=5) and Sham mice (n=5). Hippocampi were extracted one week after the injection and the ipsi et contraleral side were separated. **F.** Corresponding western blot quantification from KCC2-mCherry-BrainFectiN injected mice and Sham mice. Data are presented as median (with interquartile range), Mann-Whitney test analysis reported such as ,*: $p<0.05$; n.s : non significative, under Prism analysis.

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In such context, we were able to find in the pan-exosomes CD63-containing exosomes the 35kDa-shifted KCC2 band corresponding to the tagged KCC2 (Fig. 3A). The CNS origin was reinforced and confirmed using L1CAM immunoprecipitation of neuronal exosomes to assess the neuronal enrichment of the mCherry-KCC2 (Fig.3B). By combining the homemade KCC2 antibody, which recognizes the N-terminus part of the protein, and the mCherry antibody recognizing the mCherry molecule at the C-terminal part of KCC2 molecule we can confirm that the full-length KCC2 is embedded in exosomes and particularly neuronal exosomes as by combining the two antibodies (mCherry and KCC2), N- and C-terminus regions of the full-length KCC2 are targeted and so detected as visible by the bands located at 140 and 175kDa on figure 2.

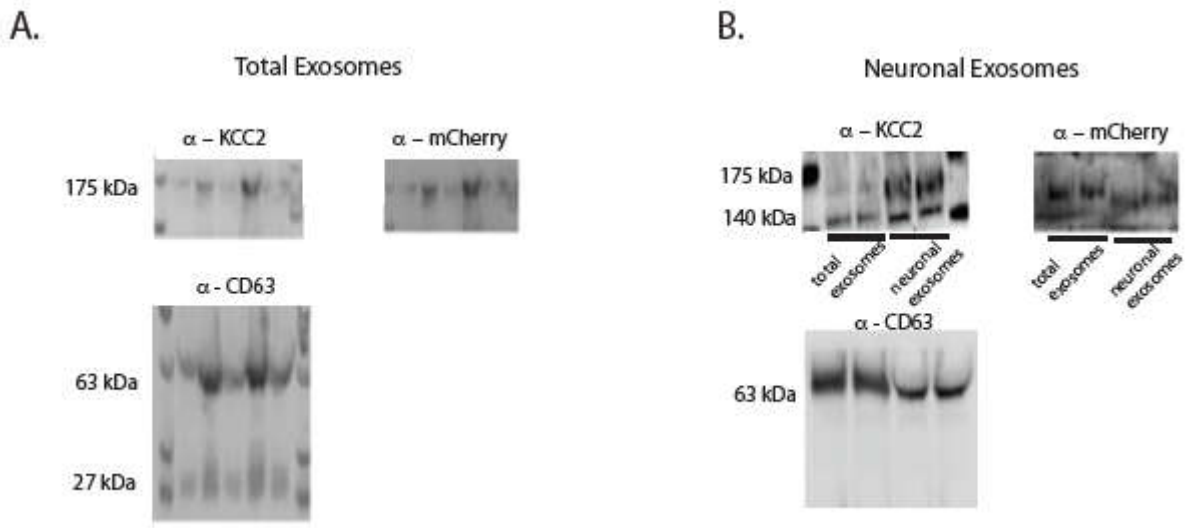

Figure 3.

3.4. Does TBI have effect on exosomes content?

Others and we have already proposed and demonstrated a transient loss of expression for KCC2 after trauma (Medina et al., 2014). Using the controlled-cortical impact model, we demonstrated a robust decrease in KCC2 over the first days after trauma with a complete recovery at one week (Goubert et al., 2019). Using the same paradigm, we followed exosomes, both total and neuronal in blood serum. We clearly observed a significant decrease in the normalized total expression of KCC2 in blood serum at 7 days post trauma (dpt)(sham 100% ± 14 vs. CCI 59,8% ± 7, *p = 0,015, n=4, Fig. 4A) and also in total exosomes (sham 100% ± 16 vs. CCI 59% ± 8, *p = 0,015, n=4, Fig. 4B), and in the neuronal-enriched fraction of exosomes (sham 100% ± 18 vs. CCI 60% ± 8, *p = 0,016, n=4, Fig. 4C). Taking into account that this loss of expression could be shifted towards longer time in blood compared to CNS expression, we did the same analysis one month after trauma. This time window was selected as we previously showed that KCC2 expression in the hippocampus recover sham expression in the same animal model and that subjects exhibit cognitive dysfunctions and more particularly depression-like behavior (Goubert et al., 2019). Here again, we observed a potent loss of KCC2 expression in blood serum (sham 100% ± 8 vs. CCI 64% ± 11, *p = 0,03, n=4, Fig. 4D), in total exosomes (sham 100% ± 8 vs. CCI 70% ± 13, *p = 0,016, n=4, Fig. 4E) and in neuronal exosomes (sham 100% ± 7 vs. CCI 70% ± 7, *p = 0,016, n=4, Fig. 4F). Thus making the hypothesis of a sustained and permanent decrease in KCC2 expression in exosomes.

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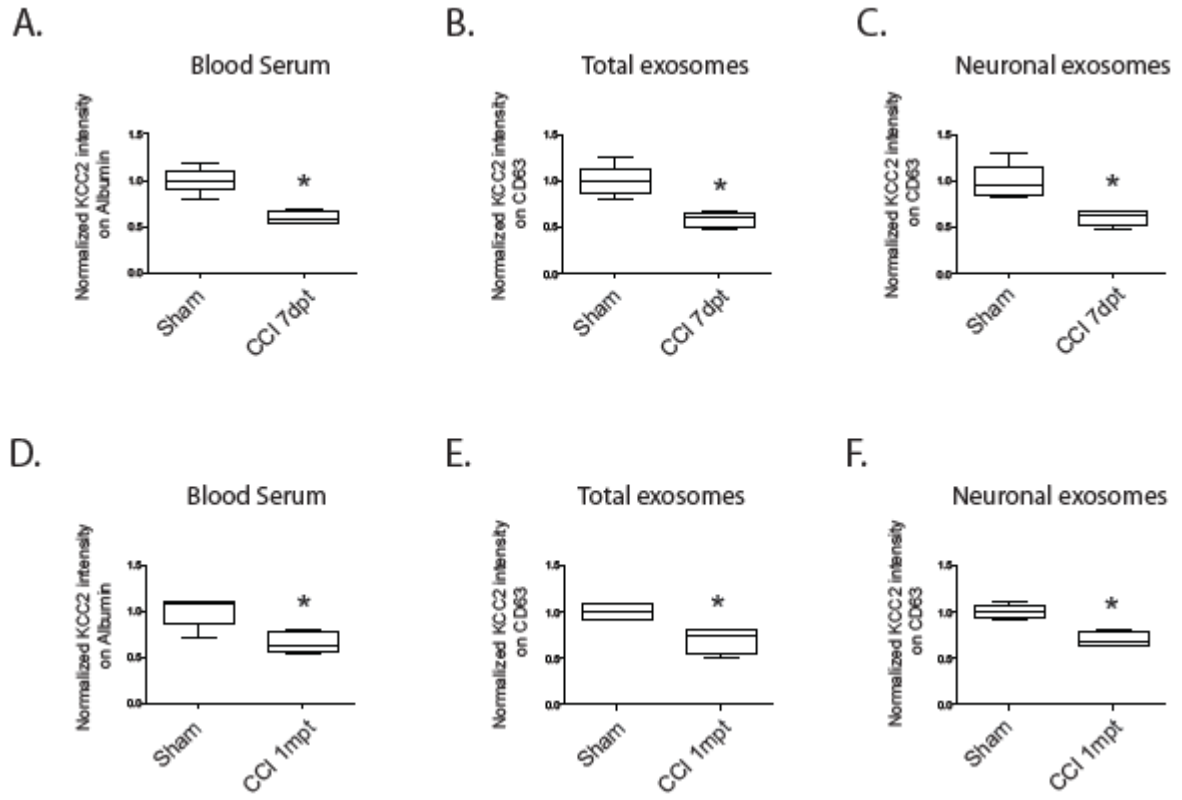

Figure 4. KCC2 expression in serum, total and neuronal exosomes in CCI condition. A. Expression of KCC2 in blood serum at 7dpt, sham versus CCI condition, n=4 animals per condition. **B.** Expression of KCC2 in total exosomes at 7dpt, sham versus CCI condition, n=4 animals per condition. **C.** Expression of KCC2 in neuronal exosomes at 7dpt, sham versus CCI condition, n=4 animals per condition. **D.** Expression of KCC2 in blood serum at 1mpt, sham versus CCI condition, n=4 animals per condition. **B.** Expression of KCC2 in total exosomes at 1mpt, sham versus CCI condition, n=4 animals per condition. **C.** Expression of KCC2 in neuronal exosomes at 1mpt, sham versus CCI condition, n=4 animals per condition. T test analysis reported such as * p<0.05; **p<0.01; ***p<0.001, under Prism analysis.

3.5. Does bumetanide have an effect on exosomes content?

Others and we already published that bumetanide, the sodium-potassium-chloride transporter antagonist has powerful effect in preventing cell death of interneurons (Goubert et al., 2019) and principal cells (Pellegrino et al., 2011) but also in preventing KCC2 rapid and transient degradation in different trauma model (Hu et al., 2017; Medina et al., 2014) and epilepsy (Kourdougli et al., 2017). We asked the question about its potential effect in KCC2 modulation in exosome content as it has been proven that this agent could efficiently restore KCC2 expression in neuronal cells. To do so, we inject during one week, intra-peritonealy twice-daily 2mg/kg of bumetanide. Again we collected blood serum and check for KCC2 expression. After such treatment, one week after trauma (7dpt), the KCC2 expression level is significantly improved compared to CCI condition and recover sham level both in blood serum (CCI 59% ± 7 vs. CCIbum 95% ± 10, *p = 0,028, Fig. 5A), in total exosomes (CCI 59% ± 8 vs. CCIbum 90% ± 9, *p = 0,028, Fig. 5B) and in neuronal exosomes (CCI 60% ± 8 vs. CCIbum 89% ± 4, *p = 0,028, n=4, Fig. 5C). The remaining question is about the long lasting effect of such agent in the persistence of KCC2 expression in link with the prevention of depressive-like behavior already observed. To assess if the treatment could modify as well the KCC2 expression in exosomes, we performed the KCC2 blood expression one month after treatment to emphasis the link in between expression and function. We observed differences in blood KCC2 expression compared to CCI on blood serum (CCI 64% ± 11 vs. CCIbum 90% ± 3, *p = 0,028, n=4, Fig. 5D), in total exosomes (CCI 70% ± 13 vs. CCIbum 1,01% ± 3, *p = 0,028, n=4, Fig. 5E) and in neuronal exosomes

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(CCI 70% ± 7 vs. CCIbum 96% ± 8, *p = 0,028, n=4, Fig. 5F). Thus making its detection potent enough to be considered as relevant in the frame of the depressive-like behavior detection. This is in link with the increase in cognitive function we already demonstrated using such approach (Goubert et al., 2019).

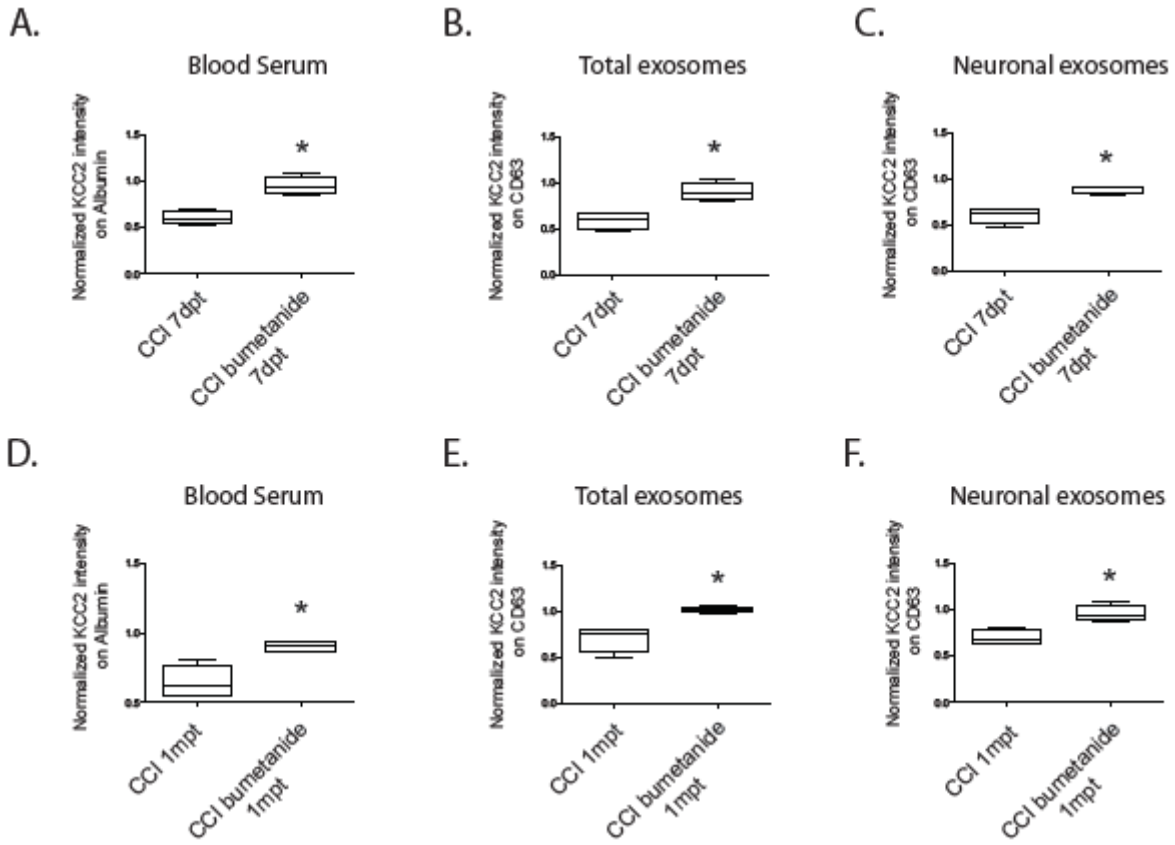

Figure 5. KCC2 expression in serum, total and neuronal exosomes in CCI condition together with bumetanide treatment. A. Expression of KCC2 in blood serum at 7dpt, CCI versus bumetanide-treated CCI condition, n=4 animals per condition. **B.** Expression of KCC2 in total exosomes at 7dpt, CCI versus bumetanide-treated CCI condition, n=4 animals per condition. **C.** Expression of KCC2 in neuronal exosomes at 7dpt, CCI versus bumetanide-treated CCI condition, n=4 animals per condition. **D.** Expression of KCC2 in blood serum at 1mpt, CCI versus bumetanide-treated CCI condition, n=4 animals per condition. **B.** Expression of KCC2 in total exosomes at 1mpt, CCI versus bumetanide-treated CCI condition, n=4 animals per condition. **C.** Expression of KCC2 in neuronal exosomes at 1mpt, CCI versus bumetanide-treated CCI condition, n=4 animals per condition. T test analysis reported such as * p<0.05; **p<0.01; ***p<0.001, under Prism analysis.

4. Discussion

Exosomes have been hypothesized to play a pathological role in several neurological disorders, particularly proteinopathies, by spreading pathological molecules to healthy tissue. Alternatively, exosomes have been postulated to play a protective role by dumping pathological molecules out of cells (Howitt and Hill, 2016; Quek and Hill, 2017). It is a quite innovative field of research as recent papers have proposed a role of exosomes in inflammation (Brites and Fernandes, 2015), miRNA regulation as well as such vesicles may transport molecules over large distance and influence the cell physiology in physiologic and pathologic context (Bagheri Hashkavayi et al., 2020; Brites and Fernandes, 2015). More recently, exosomes have been hypothesized to play important roles in the nervous system and have been shown to be involved in neurodegenerative disorders (Beach et al., 2014), axonal path finding by cell contact (Gong et al., 2016), secondary neurogenesis (Fuller et al., 2020), synaptic pruning (Bahrini et al., 2015) and network assembly (Sharma et al., 2019) and even cancer cells; however, the potential role of exosomes in normal neural

development or neurodevelopmental disorders is still unclear. Here we proposed a potential role as biomarker of the depressive-like behavior visible after brain trauma. This psychiatric disorder has a large occurrence after trauma both in the general population and in the army context (Lange et al., 2019). Having prognosis tools would be of great interest in order to prevent and to treat patient, in the last extend, biomarkers have good capacity to decide on therapeutic relevance with aspects on therapeutics efficacy that is of primary importance in the treatment of psychiatric disorders.

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