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Alginate Attenuates Reactive Microglia In Vivo

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Abstract

Alginate is a marine polysaccharide commonly used in the form of hydrogel in different biomedical applications due to its biocompatibility, low toxicity and immunogenicity, and its ability to mimic the extracellular matrix. *In vitro* studies demonstrated that alginate exerts anti-inflammatory properties, which could be exploited for targeting brain pathologies characterized by elevated levels of tissue inflammation. The present study aimed to evaluate alginate's effects on immune cells *in vivo* using the ibotenic-induced brain lesion model and the pilocarpine model of mesial temporal lobe epilepsy. The administration of calcium alginate (prepared by using the magnesium salt of alginate and calcium chloride) resulted in reduced ibotenic-associated microglial reactivity in rat brains. As a subsequent step, to enhance the translational potential of the hydrogel, lesioned epileptic rats were treated with neural stem cells and alginate; our results showed that alginate was capable of reversing the increase in Iba1 cell body size induced by neural stem cell injection, confirming that alginate exerts a modulatory effect on microglial activity.

Keywords

Animal models, Brain lesion, Ibotenic acid, Mesial Temporal Lobe Epilepsy, Microglial cells, Pilocarpine model

Introduction

Sodium alginate (Na-alginate), primarily extracted from brown seaweeds, is a linear unbranched polysaccharide composed of (1,4)-linked β -D-mannuronate and α -L-guluronate residues arranged

in homopolymeric or heteropolymeric blocks [1] [2] [3]. The high affinity of alginate chains for bivalent cations, such as Ca^{2+} , results in the formation of intramolecular cross-links, which induce alginate gelation (i.e., Ca-alginate). Modulating the concentration and the physico-chemical properties of both alginate and cross-linker enables the production of hydrogels with well-defined mechanical properties that closely replicate those of the native extracellular matrix [4] [5]. The use of alginate has been promoted by its excellent biocompatibility, low toxicity and low immunogenicity, low production cost, and mild gelation process [1]. Moreover, the physico-mechanical properties of Ca-alginate make it a valuable compound for a variety of biomedical applications, such as serving as a scaffold for neural stem/progenitor cell delivery, axonal guidance, and neural tissue repair. For instance, Ca-alginate hydrogels seeded with neural stem/progenitor cells have achieved survival, differentiation, host axon ingrowth, and partial locomotor recovery in rat spinal cord transection models [6]. Soft alginate hydrogels implanted into spinal cord lesions reduced scarring, decreased inflammation, and improved functional recovery [7]. Additionally, alginate scaffolds combined with neurotrophic factor-expressing cells guided linear axonal growth across lesion sites, enhancing nerve regeneration [8].

Notably, alginate hydrogels offer excellent control over the sustained release of low molecular weight drugs and protein therapeutics, minimizing their denaturation and preserving bioactivity [1] [9]. This controlled release capacity is particularly valuable in neuroregenerative applications, where precise delivery of neurotrophic factors, agents attenuating inflammation, or small molecules is critical for modulating the neural microenvironment and promoting repair [10] [11]. Moreover, alginate's mild gelation conditions and biocompatibility make it an ideal matrix for encapsulating sensitive molecules, ensuring their stability during delivery [12]. In the context of cell-based neural therapies, alginate not only provides a protective scaffold that supports transplanted cell survival and function, but also acts as a localized drug delivery platform that can enhance therapeutic outcomes [6] [8] [13].

Moreover, several *in vitro* studies support the hypothesis that alginate exerts anti-inflammatory and anti-oxidant effects [14] [15] [16] [17]. This aspect is relevant especially in brain pathologies characterized by a neuroinflamed environment [18] [19] [20], where increased numbers of microglia and astrocytes undergo a transition from homeostatic surveillance states to reactive phenotypes able to respond to trauma, injuries, or infections [21] [22]. Despite alginate's role being largely demonstrated *in vitro*, only a few studies investigated its role *in vivo*, with a limited focus

on spinal cord injuries and peripheral nerve lesions, while no evidence shows alginate's modulatory effect on the brain glial cells.

The present study aimed to evaluate Ca-alginate's effects on immune cells *in vivo* using i) a rat model of brain lesion induced by ibotenic acid (IBO) infusion, and ii) pilocarpine model of mesial temporal lobe epilepsy (MTLE). Ca-alginate was prepared starting from the magnesium salt of alginate (Mg-alginate), which has been reported to possess superior mechanical and biological properties compared to the commercially available Na-alginate [23] [24]. Notably, Mg-alginate has a lower viscosity (which enhances its solution injectability) and, upon calcium-mediated gelation, forms softer and less deformable matrices than those obtained from Na-alginate. Moreover, it enables a sustained release of Mg ions, contributing to its (neuro)protective effect [23] [24]. The effect of alginate has been initially assessed by examining the impact of alginate on IBO-induced brain lesions in rats; next, it has been evaluated in combination with neural stem cells (NSCs) in IBO-lesioned pilocarpine epileptic rats. The latter approach provides insight into alginate's dual role as a cytoprotective agent and a guidance scaffold in cell-based therapeutic strategies.

Results

Alginate neither reduces nor worsens IBO-induced brain lesion

A first preliminary experiment was performed to quantify the ventral *Cornu Ammonis* 3 (vCA3) bilaterally lesioned by the cytotoxic agent IBO (2 μ l). Histological analysis demonstrated a significant reduction in the percentage of intact pyramidal layer of the CA3 over total CA3 (ventral + dorsal) in IBO-lesioned compared to both sham and naïve rats (naïve: 100% [100–100], n=8 injections (inj.); sham: 90.5% [87.7–96.4], n=8 inj.; IBO-2- μ l: 66.2% [48.3–87.3], n=11 inj.; $p<0.0001$, Kruskal-Wallis; Fig. 1).

Alginate (or saline in the control group; 5 μ l volume) was infused 4 days after IBO injection, a time when acute inflammation is over, and chronic inflammation is not established yet [25]. We aimed to cover almost completely the IBO-induced lesion with alginate; therefore, IBO volume was reduced to 0.3 μ l in the following experiments. The smaller volume of IBO significantly reduced the percentage of intact CA3 compared to both sham and naïve rats (naïve: 100% [100–100], n=8 inj.; sham: 90.5% [87.7–96.4], n=8 inj.; IBO-0.3- μ l: 66.5% [45.7–74.6], n=7 inj.; $p<0.0001$, Kruskal-Wallis); the lesion sizes induced by IBO-2- μ l and IBO-0.3- μ l were similar (Kruskal-Wallis, Dunn's post-hoc: IBO-2- μ l vs IBO-0.3- μ l, $p=1$). Interestingly, the subsequent

administration of alginate in IBO-lesioned rats did not significantly change the percentage of unlesioned CA3 compared to IBO-lesioned rats receiving saline instead of alginate (IBO-ALG: 65.9% [32.7–81.3], $n=8$ inj.; IBO-sal: 68.8% [52.6–79.4], $n=7$ inj.; Kruskal-Wallis, Dunn's post-hoc: IBO-ALG vs IBO-sal, $p=1$; Fig. 1).

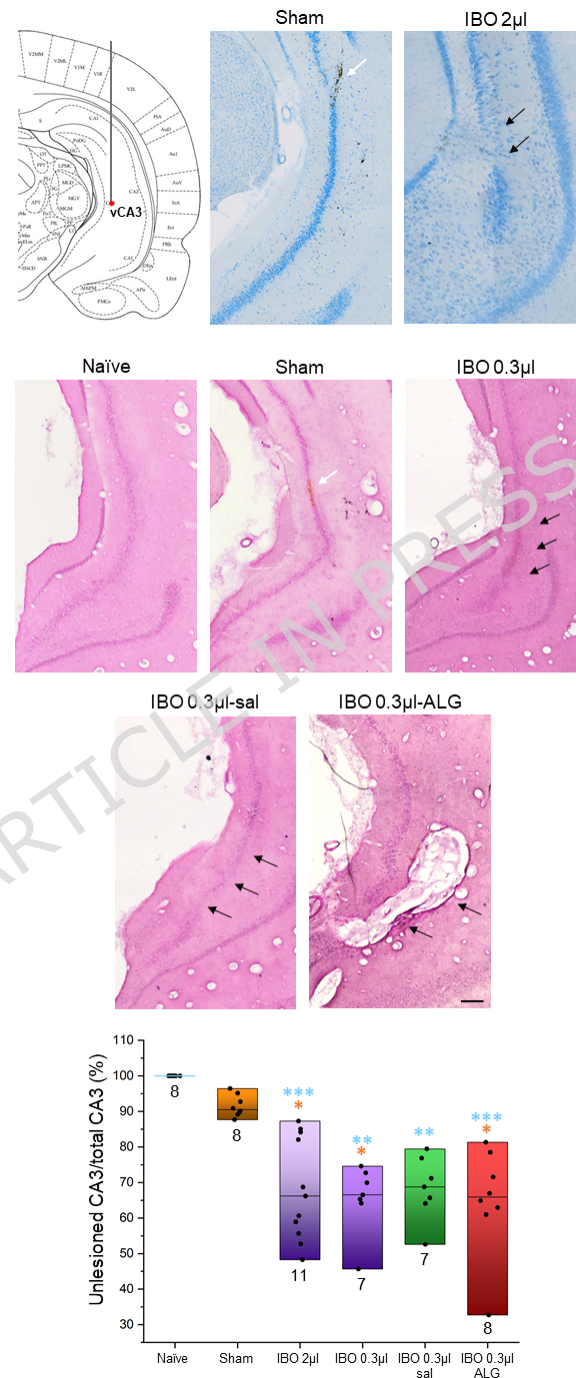


Figure 1. IBO-induced lesion in vCA3. In the top left corner, the graphic representation from the Rat Brain Atlas [26] shows the target area where the injections were performed (vCA3). Following, representative Toluidine Blue-stained

and Haematoxylin&Eosin-stained coronal sections showing the vCA3 in naïve, sham, IBO-2- μ l, IBO-0.3- μ l, and IBO-0.3- μ l followed (4 days later) by saline (IBO-sal) or alginate (IBO-ALG). White arrows indicate mechanical damage due to the infusion needle insertion in sham-operated rats. Black arrows indicate the IBO-induced lesion, characterized by CA3 pyramidal layer interruption, and, only in the IBO-ALG group, by the presence of a cavity due to alginate's higher viscosity. Scale bar: 200 μ m applies to all panels; magnification 4x. At the bottom, the box plot shows that the percentage of unlesioned CA3 (dorsal+ventral) over the total length of the CA3 is reduced in IBO-2- μ l- and IBO-0.3- μ l-lesioned rats compared to both sham and naïve rats. In addition, it shows that alginate does not affect the lesion size compared to rats receiving saline instead. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, Kruskal-Wallis followed by Dunn's post-hoc test. Data are shown as median and confidence interval (CI) [5-95]. The number of single injections is reported in the relative histogram.

From a visual perspective, animals injected with alginate appear to have a cavity, which is not observed when rats are injected with IBO alone or followed by saline. The IBO injection itself likely renders the tissue more fragile and less compact. The viscosity of alginate exerts mechanical pressure on this fragile brain tissue, causing tissue displacement that manifests as a “negative space”. In contrast, injections of saline following IBO do not generate sufficient force to displace the tissue from its original position, and therefore, it does not create a defined negative space. It is worth stressing that the IBO-induced lesion has been measured as the interruption of the CA3 pyramidal cell layer, and not as the presence of a cavity.

Alginate promotes microglial cell transition from a reactive to a surveillant state in IBO-induced brain lesion

Alginate immunomodulatory properties *in vivo* were evaluated by quantification of GFAP and Iba1 immunoreactivity (IR) mean intensity, as astrocytes and microglia reactivity are widely considered hallmarks of neuroinflammation [21] [22]. IBO injection induced a significant increase in GFAP IR mean intensity in comparison to sham-operated and naïve rats, without any significant effect of alginate treatment (naïve: 1.03 ± 0.04 A.U., $n=9$ inj.; sham: 1.83 ± 0.15 A.U., $n=4$ inj.; IBO: 6.03 ± 0.48 A.U., $n=9$ inj.; IBO-sal: 5.10 ± 0.23 A.U., $n=16$ inj.; IBO-ALG: 5.30 ± 0.43 A.U., $n=14$ inj.; $p < 0.0001$, one-way ANOVA; Fig. 2 and S2).

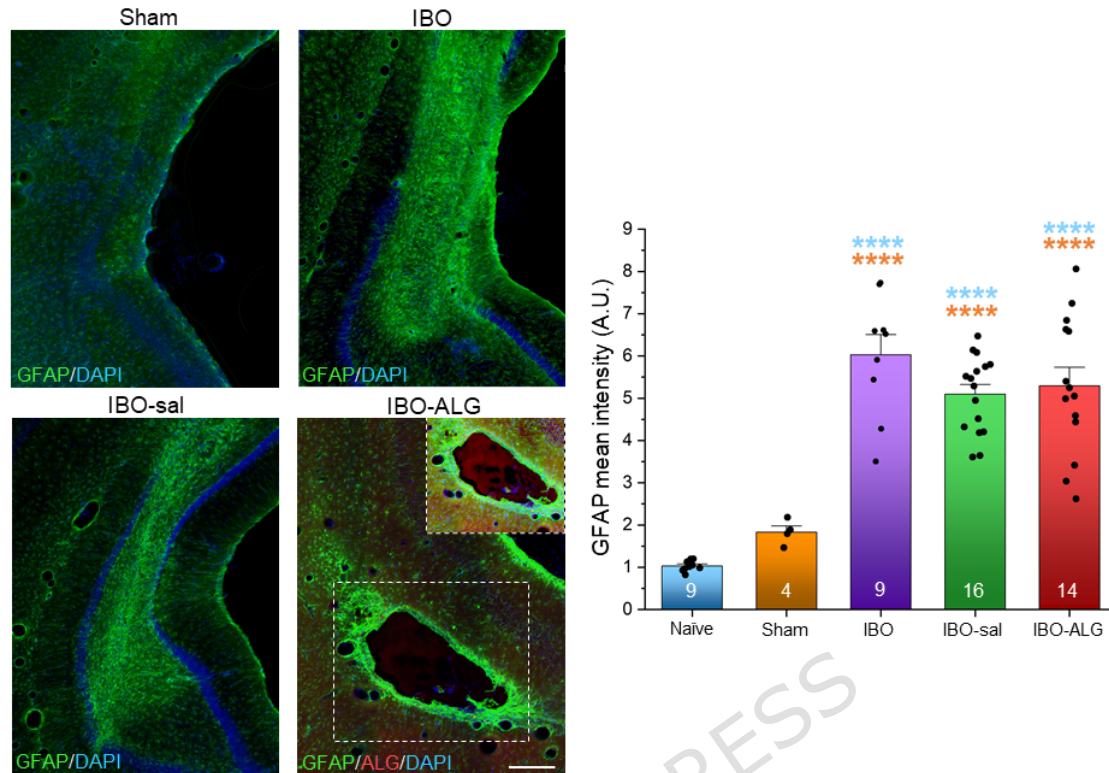


Figure 2. Alginate effect on IBO-induced astrocytosis. Representative immunofluorescence images show (i) astrocytes (GFAP, in green), and (ii) alginate (Alexa Fluor™ 568, in red); cell nuclei are counterstained with DAPI (in blue). In the inset, the exposure levels of the TRITC channel (alginate) were increased to allow better visualization of the hydrogel. Scale bar: 200 μ m applies to all panels; magnification 10x. Mean intensity was measured in 15 regions of interest (ROIs), taken in the perilesional area in the vCA3 and normalized to an internal control (see Methods section). The bar graph reveals that every group receiving IBO shows a similar increase in GFAP IR mean intensity compared to sham (orange) and to naïve (light blue) animals. **** $p < 0.0001$, one-way ANOVA followed by Tukey's post-hoc test. Data are shown as mean $\pm$ standard errors of the means (SEM). The number of single injections is reported in the relative histograms.

Accordingly, IBO injection also induced an increase in the Iba1 IR mean intensity compared to sham-operated and naïve rats, suggesting microglial reactivity as well. The subsequent administration of alginate statistically reduced Iba1 IR mean intensity, demonstrating its capability to reduce microgliosis (naïve: 0.91 ± 0.01 A.U., $n = 9$ inj.; sham: 0.79 ± 0.05 A.U., $n = 2$ inj.; IBO: 1.37 ± 0.06 A.U., $n = 9$ inj.; IBO-sal: 1.18 ± 0.06 A.U., $n = 13$ inj.; IBO-ALG: 1.05 ± 0.05 A.U., $n = 15$ inj.; $p < 0.0001$, one-way ANOVA; Fig. 3 and S2).

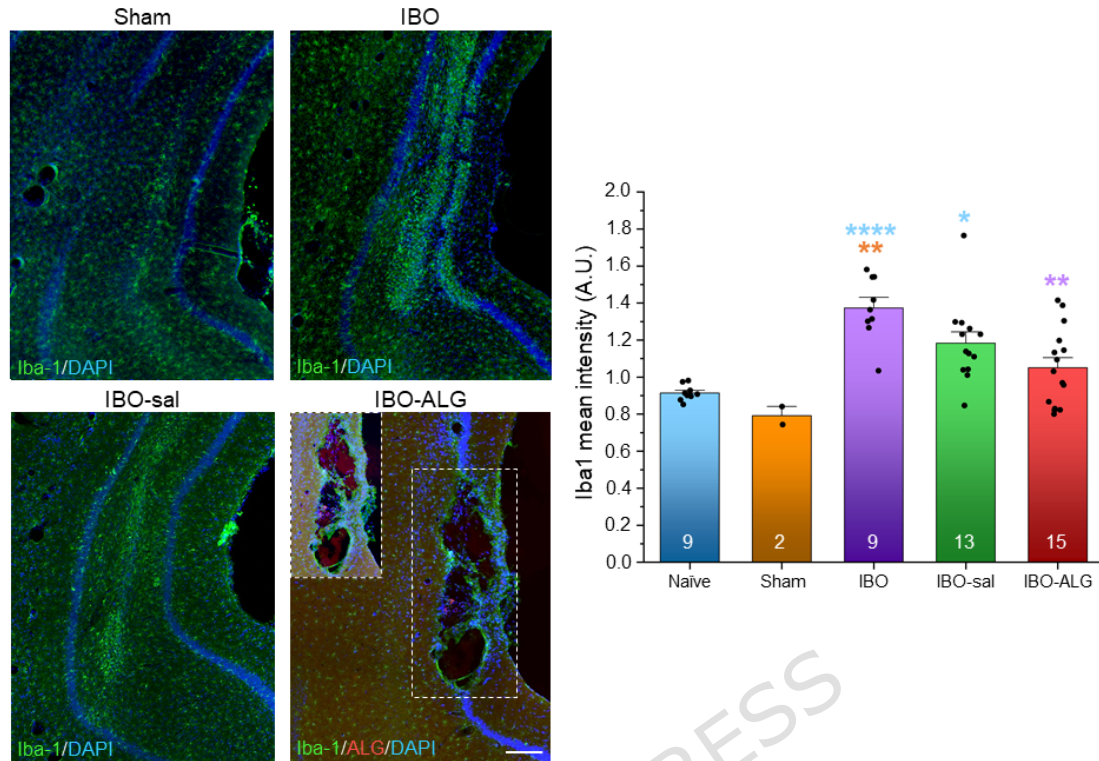


Figure 3. Alginate effect on IBO-induced microglial reactivity. Representative immunofluorescence images show (i) microglial cells (Iba1, in green), and (ii) alginate (Alexa Fluor™ 568, in red); cell nuclei are counterstained with DAPI (in blue). In the inset, the exposure levels of the TRITC channel (alginate) were increased to allow better visualization of the hydrogel. Scale bar: 200 μ m applies to all panels; magnification 10x. Mean intensity was measured in 15 ROIs, taken in the perilesional area in the vCA3 and normalized to an internal control (see Methods section). The bar graph reveals that rats receiving IBO show an increase in Iba1 IR mean intensity, compared to sham (orange) and naïve (light blue) animals; notably, alginate reduces the Iba1 IR mean intensity compared to IBO, reaching a value that is not significantly different from that observed in sham and naïve animals. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$, one-way ANOVA followed by Tukey's post-hoc test. Data are shown as mean $\pm$ SEM. The number of single injections is reported in the relative histograms.

Microglia were further investigated by assessing cell density and morphological state. Compared with sham and naïve controls, injection of IBO, alone or followed by saline, increased the density of Iba1⁺ cells (naïve: 10 cells/ROI [8.7-14.3], $n = 9$ inj.; sham: 7.8 cells/ROI [7-8.5], $n = 2$ inj.; IBO: 36.7 cells/ROI [23-65.7], $n = 9$ inj.; IBO-sal: 44.5 cells/ROI [8.5-105], $n = 13$ inj.; $p < 0.0001$, Kruskal-Wallis), while significantly reducing the proportion of surveillant (non-reactive) Iba1⁺ cells out of the total Iba1⁺ cell population (naïve: 0.9 A.U. [0.8-1], $n = 9$ inj.; sham: 1 A.U. [0.9-1], $n = 2$ inj.; IBO: 0.1 A.U. [0-0.7], $n = 9$ inj.; IBO-sal: 0.2 A.U. [0.1-0.9], $n = 13$ inj.; $p < 0.0001$, Kruskal-Wallis) (Fig. 4). Alginate administration reduced the Iba1⁺ cell density compared to the IBO-sal group (IBO-ALG: 16 cells/ROI [10-42.3], $n = 15$ inj., Kruskal-Wallis, Dunn's post-hoc: IBO-ALG vs IBO-sal, $p = 0.036$), while increasing the proportion of surveillant Iba1⁺ cells compared to both

IBO groups (IBO-ALG: 0.7 A.U. [0.5-1], $n=15$ inj., Kruskal-Wallis, Dunn's post-hoc: IBO-ALG vs IBO, $p=0.013$; IBO-ALG vs IBO-sal, $p=0.014$) (Fig. 4). These results suggest that alginate exerts its immunomodulatory effect chronically maintaining an increased surveillant activity of microglia in the perilesional area.

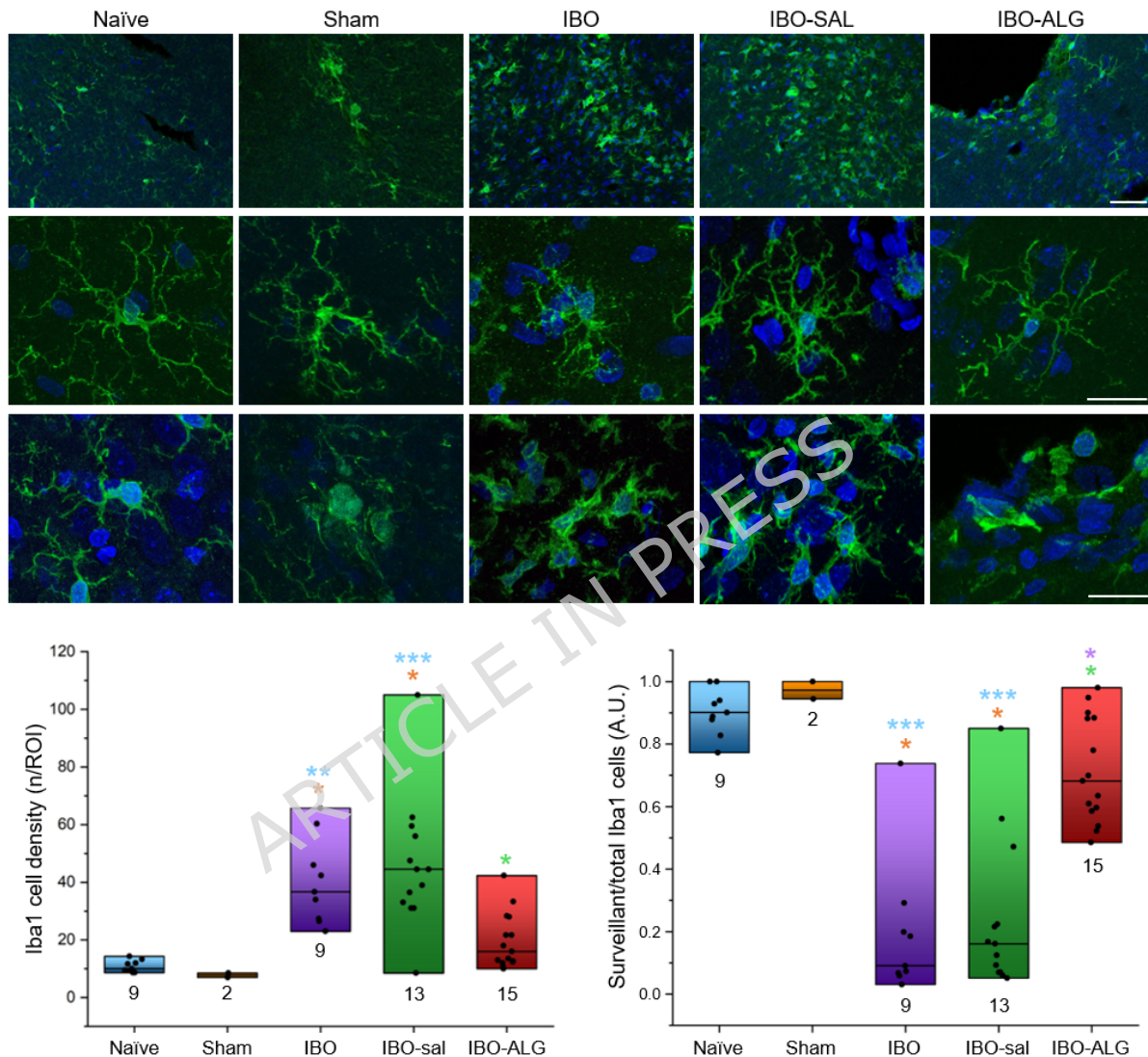


Figure 4. Alginate effect on IBO-induced microglial reactivity: cell density and morphological analyses. The top row (scale bar: 50 μm; magnification 20x) shows Iba1 immunofluorescence images representing the Iba1⁺ cell density result, while the middle and bottom rows (scale bar: 20 μm; magnification 60x) depict images representing surveillant and activated microglial morphology, respectively (Iba1 in green, nuclei in blue). Bar graphs at the bottom of the panel reveal that rats receiving IBO and IBO-sal show a significant variation in both Iba1 cell density and surveillant/total Iba1 cells, compared to sham (orange) and naïve (light blue) animals, while animals receiving alginate show variations compared to IBO-sal (green; Iba1 cell density and morphology) and IBO (purple; Iba1 morphology) groups. Data are shown as median and CI [5-95]. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ Kruskal-Wallis followed by Dunn's post-hoc test.

Behavioural tests

To assess the effects of alginate on rat behaviour, motor activity, memory task, explorative and emotional behaviour, anxiety-like behaviour, and social interaction were tested. Having demonstrated that the mechanical lesion induced by the infusion needle in sham-operated rats was negligible (not statistically different than naïve rats) in terms of histological analysis (Fig. 1), the following behavioural tests were performed only in naïve rats as the non-lesioned control group.

Motor activity

The open field (OF) test was utilized to evaluate the motor activity of the rats (Fig. 5A). Specifically, no difference in the total travelled distance was observed between the experimental groups (naïve: 39.0 ± 1.5 m, $n=5$ rats; IBO: 36.8 ± 2.5 m, $n=5$ rats; IBO-sal: 33.7 ± 3.0 m, $n=5$ rats; IBO-ALG: 36.4 ± 2.5 m, $n=5$ rats; $p=0.498$, one-way ANOVA). Furthermore, the distance travelled minute by minute was calculated to ascertain how activity levels change over the course of the test. However, the effect of the time exerted on the motor activity was found unrelated to the treatments (treatment $p=0.498$; time $p=0.005$; time*treatment, $p=0.207$, two-way ANOVA).

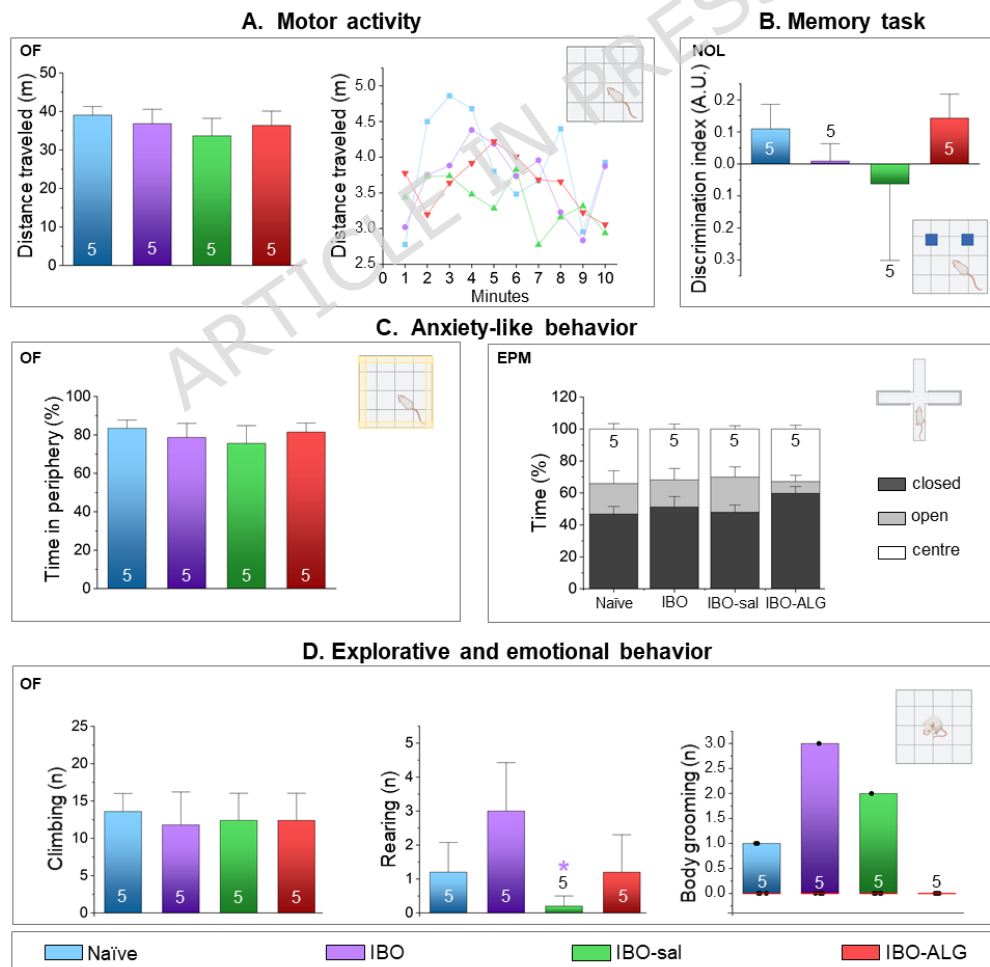


Figure 5. Behavioural tasks to test exploratory behaviour, motor activity, spatial memory, and anxiety-like behaviour. **A.** Motor activity, evaluated by the open field (OF) test, overall (left) or minute-by-minute (right), was not altered in IBO-lesioned rats compared to naïve rats; alginate did not influence the performance (left, $p > 0.05$, one-way ANOVA; right, $p > 0.05$, two-way ANOVA). **B.** Spatial memory, evaluated by the novel object location (NOL) test, was not compromised in IBO-lesioned rats, and alginate administration did not alter the performance ($p > 0.05$, one-way ANOVA). **C.** Anxiety level, assessed through the OF test and the elevated plus maze (EPM), was comparable across experimental groups, and alginate did not modify it ($p > 0.05$, one-way ANOVA). **D.** Exploratory and emotional behaviours, assessed by quantification of stereotypies, namely climbing ($p > 0.05$, one-way ANOVA), and body grooming ($p > 0.05$, Kruskal-Wallis), during the OF test, showed no differences among groups, and alginate did not alter them. On the other hand, the number of rearing declined in the IBO-sal compared to the IBO group. $*p < 0.05$, one-way ANOVA followed by Tukey's post-hoc test. Data are shown as mean $\pm$ SEM; only for body grooming (D) box plot shows median (red line) and CI [5-95]. The number of rats is reported in the relative histograms.

Spatial memory

The analysis of the novel object location (NOL) memory task revealed that IBO group showed almost no preference between familiar and novel locations of the object (discrimination index almost zero), while IBO-sal group exhibited a preference for the familiar location (negative discrimination index). When rats are injected with alginate, the discrimination index is positive, as it is in the naïve group, indicating a preference towards the novel location. However, no significant difference between the groups was detected (naïve: 0.109 ± 0.051 A.U., $n=5$ rats; IBO: 0.009 ± 0.036 A.U., $n=5$ rats; IBO-sal: -0.062 ± 0.159 A.U., $n=5$ rats; IBO-ALG: 0.143 ± 0.050 A.U., $n=5$ rats; $p=0.374$, one-way ANOVA; Fig. 5B).

Anxiety level

OF and elevated plus maze (EPM) tests were employed to evaluate the anxiety level in rats (Fig. 5C). Indeed, in OF, no significant differences emerged between groups about the percentage of time spent at the periphery of the arena suggesting no alterations in anxiety level (naïve: $83.4 \pm 2.8\%$, $n=5$ rats; IBO: $78.7 \pm 4.8\%$, $n=5$ rats; IBO-sal: $75.6 \pm 6.1\%$, $n=5$ rats; IBO-ALG: $81.5 \pm 3.1\%$, $n=5$ rats; $p=0.630$, one-way ANOVA). No changes in anxiety levels were confirmed by the measurement of the percentage of time spent in the closed arms of the EPM, which revealed no difference among groups (naïve: $46.8 \pm 4.8\%$, $n=5$ rats; IBO: $51.2 \pm 6.5\%$, $n=5$ rats; IBO-sal: $48.0 \pm 4.4\%$, $n=5$ rats; IBO-ALG: $59.7 \pm 4.3\%$, $n=5$ rats; $p=0.298$, one-way ANOVA).

Exploratory and emotional behaviour

OF was also employed to observe explorative and emotional behavioural patterns (Fig. 5D). During the test, the number of vertical behaviours (climbing and rearing) was evaluated as explorative behaviour. Despite the absence of any statistical disparities in the number of climbs among the groups (naïve: 13.6 ± 1.6 , $n=5$ rats; IBO: 11.8 ± 2.9 , $n=5$ rats; IBO-sal: 12.4 ± 2.4 , $n=5$ rats;

IBO-ALG: 12.4 ± 2.4 , $n=5$ rats; $p=0.959$, one-way ANOVA), a reduction in the number of rearings was observed in IBO-sal rats in comparison with rats that received IBO only (naïve: 1.20 ± 0.58 , $n=5$ rats; IBO: 3.00 ± 0.95 , $n=5$ rats; IBO-sal: 0.20 ± 0.20 , $n=5$ rats; IBO-ALG: 1.20 ± 0.73 , $n=5$ rats; $p=0.062$ one-way ANOVA, Tukey's post-hoc: IBO vs IBO-sal, $p=0.043$). On the other hand, the body grooming, as an indicator of emotional status, showed no differences among groups (naïve: 0 [0-1], $n=5$ rats; IBO: 0 [0-3], $n=5$ rats; IBO-SAL: 0 [0-2], $n=5$ rats; IBO-ALG: 0 [0-0], $n=5$ rats; $p=0.583$, Kruskal-Wallis).

Social interaction

Communicative behaviour was studied with the social interaction test and resident-intruder paradigm (Fig. 6). In the social interaction test our results showed that IBO-ALG animals spend significantly more time engaging with the stranger animal compared to IBO group (naïve: $46.3 \pm 4.5\%$, $n=5$ rats; IBO: $35.8 \pm 4.9\%$, $n=4$ rats; IBO-sal: $39.0 \pm 1.6\%$, $n=5$ rats; IBO-ALG: $53.3 \pm 4.7\%$, $n=5$ rats; $p=0.0398$, one-way ANOVA, Tukey's post-hoc: IBO vs IBO-ALG, $p=0.046$); however, no firm conclusion can be drawn as no difference was observed between naïve and IBO animals. The number of head entries was also evaluated, but no differences were observed among groups (naïve: 55.0 ± 5.4 A.U., $n=5$ rats; IBO: 42.5 ± 5.3 A.U., $n=4$ rats; IBO-sal: 49.6 ± 5.8 A.U., $n=5$ rats; IBO-ALG: 39.6 ± 6.1 A.U., $n=5$ rats; $p=0.253$, one-way ANOVA). In the social interaction test, one rat in the IBO group was excluded from the analysis because it unhinged the net.

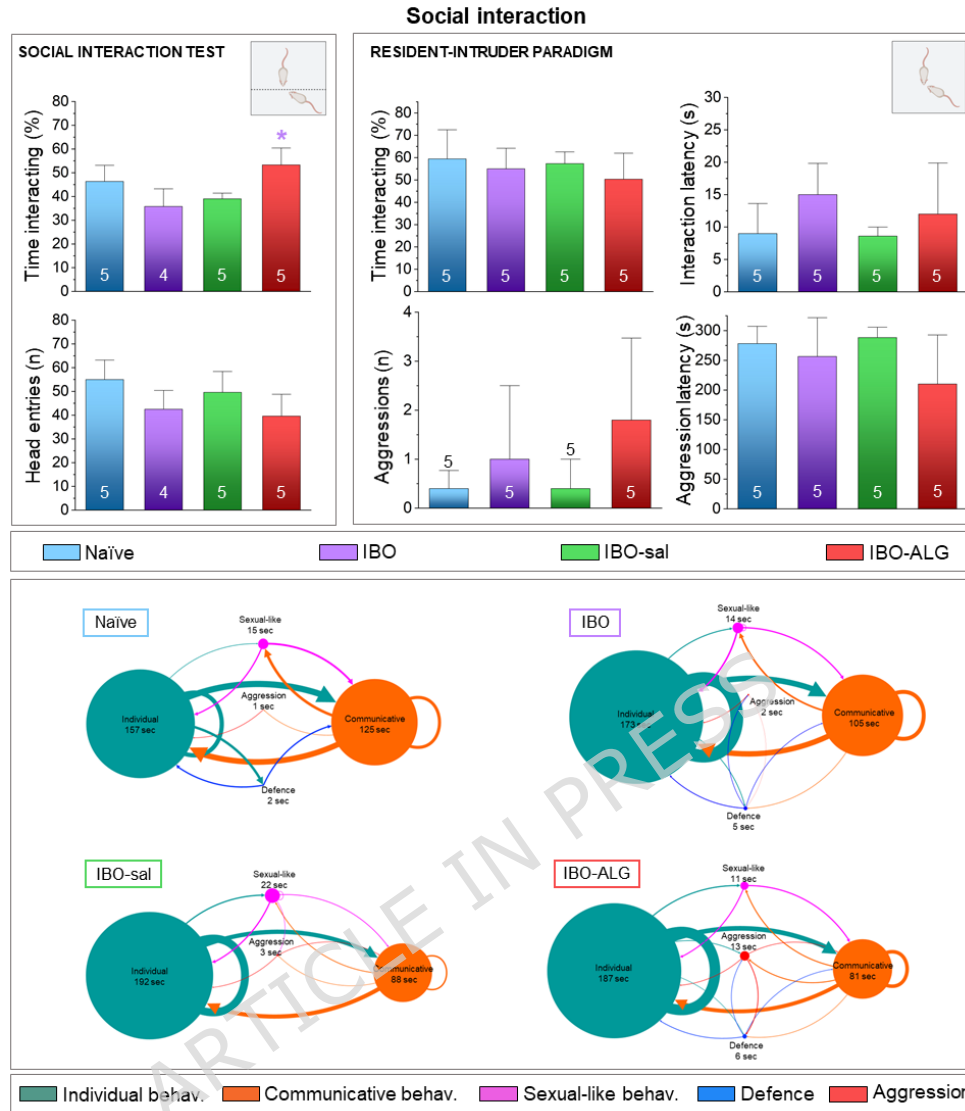


Figure 6. Behavioural tests: social interaction. The social interaction test, characterized by the presence of a net between the two interacting rats, showed that IBO-ALG rats exhibited a greater propensity to engage with an unfamiliar individual in terms of interaction time but not of interaction events ($p>0.05$, one-way ANOVA), compared to the IBO group. $*p<0.05$, one-way ANOVA followed by Tukey's post-hoc test. When the net was removed (resident-intruder paradigm), no difference was observed in terms of time spent interacting, interaction latency, number of aggressions, and aggressive behaviour latency ($p>0.05$, one-way ANOVA). Data are shown as mean $\pm$ SEM. The network graphs in the bottom panel show the time distribution among individual behaviour, communicative behaviour, sexual-like behaviour, aggression, and defence. The area of a circle is proportional to the mean duration of the time spent in each behavioural status, while the width of the arrow is proportional to the probability of transition from one type of behavioural status to another. The times indicated denote the total mean time spent engaging in a particular behavioural status.

In the resident-intruder paradigm the time spent interacting (naïve: $59.5 \pm 8.7\%$, $n=5$ rats; IBO: $55.0 \pm 6.1\%$, $n=5$ rats; IBO-sal: $57.3 \pm 3.5\%$, $n=5$ rats; IBO-ALG: $50.3 \pm 7.8\%$, $n=5$ rats; $p=0.802$, one-way ANOVA) and the latency to interaction (naïve: 9.00 ± 3.08 s, $n=5$ rats; IBO: 15.00 ± 3.21 s, $n=5$ rats; IBO-sal: 8.60 ± 0.93 s, $n=5$ rats; IBO-ALG: 12.00 ± 5.24 s, $n=5$ rats; $p=0.544$, one-way

ANOVA) were calculated. Our results revealed no significant change in both parameters among groups, suggesting no alteration in the social behaviour. Despite 2 out of 5 naïve and IBO-ALG rats exhibited aggression compared to 1 out of 5 IBO and IBO-sal rats, we did not find any significant difference in aggression level among groups (naïve: 0.40 ± 0.24 , $n=5$ rats; IBO: 1.00 ± 1.00 , $n=5$ rats; IBO-sal: 0.40 ± 0.40 , $n=5$ rats; IBO-ALG: 1.80 ± 1.11 , $n=5$ rats; $p=0.557$, one-way ANOVA), nor in the latency to the first attack (naïve: 278 ± 19 s, $n=5$ rats; IBO: 257 ± 43 s, $n=5$ rats; IBO-sal: 288 ± 12 s, $n=5$ rats; IBO-ALG: 210 ± 55 s, $n=5$ rats; $p=0.467$, one-way ANOVA). We further studied the social behaviour in the resident-intruder paradigm, describing five categories belonging to different behavioural patterns: communicative, individual, sexual-like, aggression, and defence. The most prevalent behaviours observed across all experimental groups pertained primarily to individual and communicative behaviours. These were followed by sexual behaviours and, to a lesser extent, aggressive and defensive behaviours. The study of the interplay among social behaviours did not reveal an obvious impairment in the social behavioural programming in any group.

***In vivo* alginate attenuates NSC-induced changes in microglial cell body size**

It has been demonstrated that alginate is able to promote the differentiation of NSCs *in vitro* [27]. Intracerebral injection of NSCs is one of the alternative therapeutic interventions under investigation to treat several brain disorders characterized by the presence of a lesion, including MTLE [28]. In our laboratory, we performed intracerebral injection of NSCs, alone or in combination with alginate, in the rat pilocarpine model of MTLE. In these rats, IBO was injected 4 days before NSCs or NSCs + alginate infusion to create a cavity, preventing an increase in intracranial pressure, and to locally erase epileptic cells, avoiding interaction of NSCs with endogenous cells characterized by a pathological phenotype. Our experiments represent, to the best of our knowledge, the first *in vivo* investigation of the influence of alginate on host glial responses in pathological brain conditions.

Glial scar thickness was measured and found not significantly different among groups (IBO: 75.4 ± 0.8 μm , $n=2$ inj.; IBO-ALG: 62.3 ± 4.2 μm , $n=5$ inj.; IBO-NSC: 84.7 ± 8.0 μm , $n=6$ inj.; IBO-NSC-ALG: 70.7 ± 5.4 μm , $n=13$ inj.; $p=0.223$, one-way ANOVA; Fig. 7).

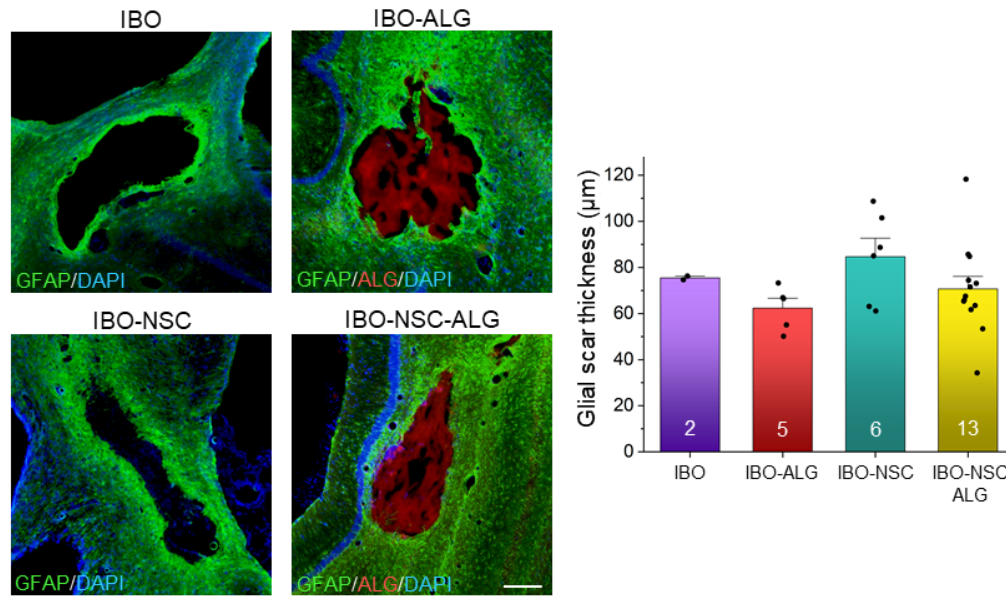


Figure 7. Alginate effect on glial scar in IBO-lesioned epileptic rats. Representative immunofluorescence images show glial scar (GFAP IR, in green) around the lesion site, and alginate (Alexa Fluor™ 568, in red); cell nuclei are counterstained with DAPI (in blue). Scale bar: 200 μm applies to all panels; magnification 4x. The bar graph reveals that alginate does not affect the glial scar thickness ($p > 0.05$, one-way ANOVA). Data are shown as mean $\pm$ SEM. The number of single injections is reported in the relative histograms.

The analysis of Iba1 IR revealed no differences in the Iba1 cell density among groups (not lesioned: 14.0 cells/ROI [9.3–20.9], $n=5$ inj.; IBO: 14.5 cells/ROI [10.3–15.2], $n=3$ inj.; IBO-ALG: 18.8 cells/ROI [18.3–19.3], $n=2$ inj.; IBO-NSC: 26.0 cells/ROI [13.5–31.5], $n=3$ inj.; IBO-NSC-ALG: 12.5 cells/ROI [11.3–27.7], $n=6$ inj.; $p=0.290$, Kruskal-Wallis; Fig. 8). NSC infusion in lesioned epileptic rats induced an increase in microglial cell body size compared to lesioned and unlesioned tissue not receiving NSCs (Fig. 8); interestingly, when co-infused with NSCs, alginate was associated with microglial cell body sizes comparable to those observed in the control, IBO, and IBO-ALG groups (not lesioned: $15.7 \pm 0.6 \mu\text{m}^2$, $n=5$ inj.; IBO: $14.7 \pm 1.4 \mu\text{m}^2$, $n=3$ inj.; IBO-ALG: $15.1 \pm 0.5 \mu\text{m}^2$, $n=2$ inj.; IBO-NSC: $27.6 \pm 2.4 \mu\text{m}^2$, $n=3$ inj.; IBO-NSC-ALG: $16.2 \pm 1.4 \mu\text{m}^2$, $n=6$ inj.; $p=0.0003$, one-way ANOVA, Tukey post-hoc: IBO-NSC vs not lesioned, $p=0.0004$; IBO-NSC vs IBO, $p=0.0006$; IBO-NSC vs IBO-ALG, $p=0.0023$; IBO-NSC-ALG vs IBO-NSC, $p=0.0005$). These findings suggest that alginate may attenuate local microglial morphological changes induced by NSC transplantation. However, since alginate did not improve graft survival or reduce glial scar thickness, the present data do not allow us to conclude that alginate exerted a functional restorative effect. The observed reduction in microglial cell body size may alternatively reflect a partial physical isolation of transplanted NSCs from the host tissue, thereby limiting direct interactions between NSCs and host microglia.

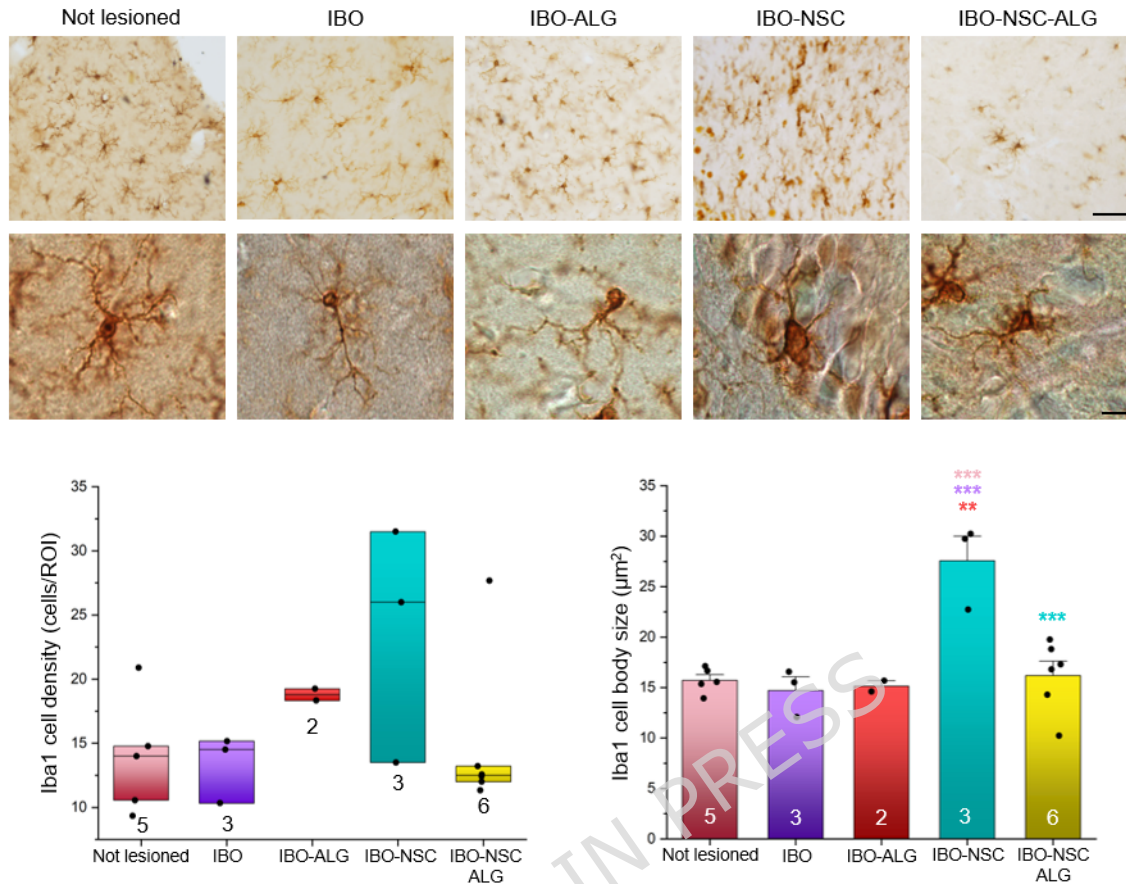


Figure 8. Alginate effect on IBO-induced microglial reactivity. The top row (scale bar: 50 μm; magnification 20x) shows Iba1 immunohistochemistry images representing the cell density result, while the bottom row (scale bar: 10 μm; magnification 100x) depicts images representing the microglial cell body. Bar graphs at the bottom of the panel show no significant variations in terms of Iba1 cell density between groups (left; $p > 0.05$, Kruskal-Wallis). Data are shown as median and CI [5-95]. On the other hand, rats receiving NSCs displayed, on average, an increase in microglial cell body size (right), which resulted reduced when alginate was added to the mix. Data are shown as mean $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$ one-way ANOVA followed by Tukey's post-hoc test, compared to not lesioned (pink), IBO (purple), IBO-ALG (red), IBO-NSC (green).

Discussion

Although *in vitro* studies have shown anti-inflammatory effects of alginate [14] [15] [16], these properties *in vivo* remained poorly investigated. Our study aimed to evaluate alginate's immunomodulatory potential *in vivo* using two animal models characterized by neuroinflammation: a rat model of brain lesion induced by IBO infusion and the pilocarpine model of MTLE.

Previous work has shown that alginate hydrogels formulated within the concentration range used here display minimal swelling after gelation. Palazzolo et al. [29] reported no significant volumetric expansion, indicating that these hydrogels remain stable once crosslinked. This

property is particularly important for deep brain injections, as post-injection swelling could increase local pressure and risk mechanical damage to surrounding neural tissue. The limited swelling behaviour of this material, therefore confirms the suitability of our hydrogel formulation for safe intraparenchymal delivery.

Our results showed that brains lesioned by IBO injection displayed an increased glial response quantified by GFAP and Iba1 IR mean fluorescence intensity. These findings are consistent with what has been observed in other models of brain lesion, such as the exposure to a mechanical brain lesion [30] [31] and intracerebral injection of kainic acid [32]. Our study showed that alginate reduces Iba1 mean intensity while favouring microglial cell transition from reactive to surveillant state in lesioned brains, without affecting lesion-induced astrogliosis, despite the mechanical displacement of tissue caused by hydrogel injection. If the observed effects were primarily due to mechanical injury associated with the viscosity of the material, an increase in glial reactivity would be expected [33]. Therefore, the reduction in microglial reactivity is unlikely to be explained solely by the mechanical displacement of the tissue. This result is aligned with the study by Grulova and colleagues [10], who reported that the administration of alginate in rats following spinal cord injury decreased Iba1 expression but failed to reduce astrogliosis. This differential response likely reflects the distinct roles and sensitivities of microglia and astrocytes within the central nervous system. Microglia act as primary immune sentinels and readily react to subtle changes in the chemical or structural environment [34]. Alginate can modulate microglial behaviour through interactions with pattern-recognition receptors or by altering local ionic or biochemical conditions. In contrast, astrocytes typically require stronger stimuli, such as mechanical injury, cytokine signalling, or blood–brain barrier disruption, to become reactive [35]. Given alginate's inert and non-adhesive properties, it is unlikely to reach the reactivity threshold for astrocytes, explaining why astrogliosis remained unchanged despite measurable effects on microglia. Further characterization of microglial cells, such as the M1/M2 phenotype polarization, has not been performed, as the binary classification (M1 microglia promoting neuroinflammation and neurotoxicity, and M2 microglia exerting anti-inflammatory and neuroprotective effects) has been increasingly recognized as an oversimplification [21].

Several studies have previously shown that injection of GABAergic progenitors induced a reduction in seizure frequency but failed to restore cognitive impairments in animal models of epilepsy [36] [37]; this is most likely due to the fact that the mechanisms underlying cognition,

such as long-term potentiation, are based on synaptic plasticity of glutamatergic excitatory neurons [38]. To overcome this problem, we attempted an alternative intervention using NSCs, able to differentiate into both excitatory and inhibitory neurons, in addition to astrocytes [39]. Our approach is further challenging due to the previous injection of the cytotoxic agent IBO (4 days earlier), to create a cavity and prevent an increase in intracranial pressure, but mostly to avoid contamination of injected NSCs with endogenous cells owning a pathological phenotype. Unfortunately, only sparse NSCs reached a mature phenotype, although nearly all slices were analysed for NeuN positivity to ensure no injected cells were missed, and their number was not sufficient to be clinically relevant (data not shown). In accordance with many other studies, stem cell survival after transplantation is extremely critical, especially in pathological environments [40] [41] [42]. Survival and differentiation of transplanted NSCs were beyond the main scope of the present work, which aimed to investigate the immunomodulatory effects of alginate *in vivo* in both lesioned and lesioned epileptic brains. However, it is worth to note that the transplantation of stem cells that failed to mature and to survive over time likely contributes to increase inflammation in the microenvironment (Fig. 8). Although alginate did not improve the differentiation and survival of the transplanted cells, its presence still helped modulating the local immune environment, particularly acting on the microglial cells, as indicated by the reduction in cell body size (Fig. 8). Nevertheless, we cannot exclude the possibility that this reduction may result, at least in part, from the partial physical isolation of injected cells within the alginate matrix [43] rather than solely a direct modulation of microglial activity. NSCs drastically increased the size of the Iba1⁺ IR cell body, and this was in line with what was observed in a murine model of Alzheimer's disease treated with human NSCs, in which Iba1⁺ IR cells exhibited a reactive morphology characterized by large amoeboid cell bodies [44]. Alginate, when co-injected with NSCs, significantly decreased microglia cell body size. Interestingly, this effect was observed only when inflammation was exacerbated by NSC injection, while it was not evident in epileptic brains receiving alginate alone. Previous work demonstrated that Mg-alginate protects NSCs from oxidative stress and reduces their production of reactive oxygen species [23]. This antioxidant property may contribute to its ability to modulate microglial activity, as observed in the present study; however, direct investigation of inflammatory pathways would be required to confirm this effect.

Alginate hydrogels have become widely used in cell delivery owing to their biocompatibility, mild gelation conditions, and ability to create protective three-dimensional microenvironments that preserve cell viability during transplantation [45]. Their tunable mechanical properties allow injection through fine needles while maintaining structural integrity, making them particularly suitable for minimally invasive procedures such as deep brain delivery, and therefore attractive for NSC transplantation. However, only a few studies have explored alginate specifically as a vehicle for NSCs. For example, an oxidized alginate–silk fibroin hydrogel supported high NSC survival and functional recovery in a rat model of cerebral palsy [46], while a gelatine/alginate scaffold improved NSC retention in traumatic brain injury models [43]. The present work establishes Mg-alginate as a safe vehicle since it does not exacerbate glial cell responses; on the contrary, it reduces microglial reactivity when injected with NSCs, providing a simple and preclinically realistic delivery platform. Building on this foundation, future studies should explore whether introducing controlled degradability (e.g., matrix metalloproteinase-cleavable peptides) or cell-adhesive cues (e.g. arginyl-glycyl-aspartic peptides) could further support long-term engraftment and integration within the brain, enabling systematic comparison between native and engineered alginate systems to define the material properties most critical for durable neural repair.

In our study, injections targeted the vCA3, a region critically lesioned in MTLE [47], and that can be a potential target of future therapeutic interventions. The injection of cytotoxic agents into the vCA3 has been demonstrated to induce cell loss and neuroinflammation, which may consequently lead to cognitive impairments, social stress, and anxiety [48] [49] [50] [51]. In accordance with another study [52], our data show that rats lesioned in the ventral hippocampus have no deficits in spatial working memory, a task that relies on the dorsal portion of the hippocampus rather than the ventral subregion [53]. We did not observe impaired social behaviour in our lesioned rats, most likely because the IBO-induced lesion was extremely minimal and localized to a portion of vCA3, while the ventral hippocampus remaining intact was sufficient to somehow compensate for the damaged area. Therefore, our study shows alginate effects at the cellular level, while clear functional effects could not be identified in this specific experimental setup. Accordingly, no conclusions regarding behavioural efficacy are drawn.

Carprofen (5 mg/kg), an anti-inflammatory agent used to reduce rat discomfort and acute inflammation, was administered in all surgeries, and its effect was expected to last no more than 24 h. The analysis has been performed in the chronic condition, several weeks after the surgery (5-

30 weeks post-IBO injection), and we do not expect the results to have been affected by carprofen administered several weeks earlier. In addition, sham-operated rats, used as the control group, received Carprofen as well.

Limitations and Conclusion

One of the limitations of the present study is the small sample size in the experimental groups, mainly due to the challenge faced in intracerebral injections in deep brain structures, and future studies on larger experimental groups should confirm our results. Specifically, a minimum brain size variability among rats, and the not perfectly straight insertion of the infusion needle, may lead to errors that are amplified in the deep brain areas. In addition, the viscosity of alginate, along with its fluid-to-hydrogel transition within the tissue, had to face the pressure exerted by the surrounding tissue. This led, in some cases, to the formation of the matrix towards the interthalamic space, missing in some cases the real target area. Rats in which injections were mislocated were more frequent than expected in the epileptic group, where tissue is more fragile, and structural deformation caused by ventricular dilatation and hippocampal atrophy increased the risk of injecting in the wrong area [54]; these rats were excluded from the study.

This study relies mainly on IR mean intensity and morphological indicators, whose reduction suggests a change in the microglial state. Complementary molecular analyses of inflammatory cytokines, genes, or signalling pathways would further strengthen the conclusions. However, due to the small and highly localized nature of the lesion, detecting appreciable changes would require analysis restricted to isolated perilesional tissue, which is not technically feasible. Moreover, despite the use of 70 bilaterally injected animals, the number of available sections remained inherently limited due to the small lesion area and technical challenges associated with the injections. Consequently, all sections were fully allocated to the planned histological analyses.

Another noteworthy aspect is the inherent alginate batch-to-batch variability in fluorescence signal intensity. In some experimental batches, the TRITC signal (AF547) required increased exposure to allow qualitative visualization of the alginate hydrogel and confirm its localization within the tissue (inset of Figures 2, 3, and S2). Importantly, to minimize potential bias associated with these variations, all quantitative analyses were performed exclusively on non-overexposed images and were restricted to the FITC channel for mean intensity assessment.

To conclude, our study suggests that alginate exerts an immunomodulatory influence *in vivo*, reflected by changes in microglial density, morphology, and reactive state. In lesioned brains,

alginate results associated with reduced microglial reactivity and promotes a shift towards a more surveillant microglial phenotype, while attenuating the increase in microglial cell body size observed following NSC transplantation, possibly through partial physical isolation of transplanted cells from the host tissue. Additional molecular investigations will be necessary to further characterize the underlying mechanisms and assess the functional significance of these observations.

Methods

Animal ethical statement

All experiments on animals were performed strictly in accordance with the European Directive 2010/63/EU and the Use of Care of Laboratory Animals, as well as the ARRIVE guidelines. The protocols approved by the Animal Welfare Body of the University of Modena and Reggio Emilia (UNIMORE) and of the University of Verona (UNIVR) were then authorized, respectively, by the Italian Ministry of Health (54/2019-PR) and by the Italian National Institute of Health (C46F4.N.N4E) according to the national guidelines on animal experimental research (art.31 legislative decree 26/2014). All efforts were made to refine the procedures, improve animals' welfare, and reduce the number of animals used, complying with the 3Rs.

Experiments were conducted on adult Sprague Dawley rats purchased from Charles River Laboratories (Italy). Animals were housed in standard cages provided with environmental enrichment, under controlled conditions: temperature 22 ± 1 °C, humidity about 60%, with a 12h light/dark cycle, and free access to food and water. Following an acclimation period of several days, rats were handled to familiarize them with the researchers and to minimize stress associated with the experimental procedures [55].

Experimental plan

Forty-four male rats (201-225 g) were employed to evaluate alginate immunomodulatory properties in lesioned rats, while twenty-six male rats (70-100 g) were used to study alginate properties in epileptic brains in combination with NSC treatment.

For experiments on lesioned (non-epileptic) rats, 1 week after animals' arrival, rats underwent IBO intracerebral injection through 26s-gauge (G) needles inserted bilaterally to reach the targeted area of the vCA3. The second injection of saline or alginate, when needed, was performed 4 days later. Animals' weight was recorded weekly to monitor their general health condition until animal

perfusion (9-30 weeks post-injection); lesioned rats did not show any decrease in body weight (two-way ANOVA for repeated measures: treatment $p=0.858$; Supplementary materials, Fig. S1). For experiments on epileptic lesioned rats, 1 week after the animals' arrival, rats were injected with pilocarpine to induce *Status epilepticus* (SE). After two weeks of recovery, rats underwent stereotaxic surgery for bilateral cannula implantation to perform multiple injections, avoiding long periods under anaesthesia, which could lead to respiratory failure in epileptic subjects [54]. The infusion procedures were conducted by inserting 27 G needles through the cannulas, 10 (IBO) and 14 (alginate, NSCs, alginate+NSCs) days after their implantation. Rats were intracardially perfused at 5-9 weeks post-injection.

Pilocarpine model of MTLE

The pilocarpine model [56] [57] was used to induce MTLE. Intraperitoneal (ip.) administration of pilocarpine hydrochloride (380 mg/kg; P-6503, Merk, Germany) was preceded 30 min earlier by injection of scopolamine methyl nitrate (1 mg/kg, ip.; S-2250, Merk, Germany) to reduce peripheral cholinergic adverse effects. A few minutes after pilocarpine treatment, seizures started occurring, and their severity increased over time until they culminated in SE. Seizures were scored through a modified Racine's scale [58]: stage 0, no observable behavioural changes; stage 1, mouth and facial automatisms, and immobility; stage 2, head tremors, body automatisms, and involuntary movements; stage 3, limb clonus, jerks, and spasms; stage 4, rearing, tonic-clonic convulsions without loss of posture, and tail stiffening; stage 5, rearing and falling, and tonic-clonic convulsion with loss of posture; stage 6, SE. Thirty min after SE onset, rats were injected with a combination of ketamine/diazepam (20/4 mg/kg, ip.) to stop convulsive manifestations. After the procedure, animals were monitored for up to 4 hours (h), and were subjected to frequent observation for at least 3 days until their complete recovery. Only rats experiencing SE were included in the study, while those not experiencing SE were excluded.

Stereotaxic surgeries

Before utilization, surgical instruments were sterilized with 70% ethanol for 30 minutes (min). The animals were weighed and anesthetized with isoflurane (4% at induction in the box, 1.5-2% for maintenance at mask, delivered at a flow rate of 1.5 l/min). An anti-inflammatory medication (Carprofen 5 mg/kg) was then administered subcutaneously (sc.) to alleviate distress. The animals were subsequently secured within the stereotaxic apparatus (David Kopf Instruments, USA) and, after shaving the head and applying povidone-iodine 10% (Betadine) antiseptic solution, a local

anaesthetic (Lidocaine 2%, 200 μ l, sc.) was administered. Then the scalp was incised, and periosteum removed to perform bilateral craniotomies (0.9 mm) with a precision drill at the following stereotaxic coordinates: anteroposterior (AP): -5.5 mm from Bregma; mediolateral (ML): $\pm$ 4.5 mm from midline for rat weigh $\leq$ 285 g, and $\pm$ 4.8 mm from midline for rat weigh $>$ 285 g [54] extrapolated from the Rat Brain Atlas [59].

Intrahippocampal infusions

In non-epileptic animals, infusions were carried out through two needles (26s G) connected with polyethylene tubes to two Hamilton syringes (10 μ l Gastight 1801 RN, Hamilton Company, Switzerland) placed on an infusion pump (Legato, KD Scientific Inc., USA). Once the syringes were loaded and the needles were fixed to stereotactic holders, dura mater was cut, and the needles were lowered with a micromanipulator to reach vCA3 at dorsoventral (DV) coordinate of -5.5 mm from dura mater. After the infusions (1 μ l/min speed), needles were left in place 5 min to avoid backflow and, finally, they were gently extracted and the rat head was sutured.

In epileptic animals, intrahippocampal injections were performed through cannulas which were implanted as described previously [54]. A week after cannulas' implantation, animals were briefly anesthetized to perform infusions using the system described above. A 27 G infusion needle, whose length was adjusted to reach the vCA3 (DV -5.5 mm from dura mater), was slowly inserted manually in each cannula. After the infusions (1 μ l/min), needles were left in place for 5 min, and then they were slowly removed.

To mitigate the discomfort associated with surgical procedures and the risk of infection, anti-inflammatory (Carprofen 5 mg/kg, sc.) and antibiotic (Enrofloxacin 5 mg/kg, sc.) drugs were administered at the end of the surgeries. A gel containing anaesthetic and antimicrobial agents (Lidocaine 2.50 g, Neomycin 0.50 g, Fluocinolone 0.025 g) was applied topically over the surgical incision. After the surgeries, animals are transferred into clean cages and kept under observation. The pain level was monitored for the following 3 days through the rat Grimace Scale [60].

Rats were randomly assigned to different experimental groups, fully detailed in Supplementary materials (Tables S1 and S2). Sham animals underwent the same surgical procedure, with infusion needles simply being inserted and removed after 5 min.

IBO

IBO (Tocris, Bio-Techne SRL, USA) powder was reconstituted with sterile PBS and sonicated for 3 min to achieve a final concentration of 1 mg/ml at pH 7.4. The stock solution was then aliquoted and stored at -20 °C. On the day of injection, one aliquot was thawed and used immediately.

Alginate

Materials. Na-alginate was purchased from DuPont Nutrition Norge – NovaMatrix (Norway). Mg-alginate was obtained starting from Na-alginate *via* an ion exchange mechanism with MgCl_2 , as described previously [23]. Briefly, Na-alginate was dissolved in MilliQ water, dialyzed against 0.25 M MgCl_2 and then against MilliQ water adjusted to pH 8.0 with $\text{Mg}(\text{OH})_2$. Elemental analysis using inductively coupled plasma optical emission spectroscopy (ICP-OES) revealed that Mg-alginate contained approximately 1 mmol of magnesium per gram of polymer, corresponding to one magnesium ion per 4.6 carboxyl groups (degree of ionization about 44%). At alginate concentrations of 0.2–0.4 mg/ml, the magnesium concentration was close to 2.0 mM, similar to physiological levels in the brain [61]. Fluorescent alginate was prepared by functionalizing alginate with Alexa Fluor™ 568 (ThermoFisher Scientific, USA) [24].

Method. Injectability tests were performed *in vitro* by rapidly mixing Na-alginate or Mg-alginate with calcium chloride (CaCl_2) in 0.9% NaCl or NaCl/Hepes [135/20 mM] buffer or MilliQ) and injecting using a syringe pump equipped with 28 G or 33 G needles. Alginate is injected through needles decreasing in size (with increasing gauges) to test the minimum diameter while avoiding needle obstruction. Our results showed that Mg-alginate showed better injectability compared to Na-alginate (Supplementary materials, Table S3). Indeed, no material occlusion occurred in the needle when injecting Mg-alginate with CaCl_2 concentrations up to 1 mM, through the smaller-gauge needle (33 G). In contrast, when injecting Na-alginate with 1 mM CaCl_2 using the same needle, flow was hindered and partial blockage was observed. Based on these findings and those reported in [23] [24], Mg-alginate was selected for the *in vivo* tests to evaluate its immunomodulatory properties.

NSCs

At the University of Verona, pregnant female Sprague Dawley rats were anesthetized using isoflurane 4% and sacrificed by cervical dislocation on the 18th day of gestation. The embryos were extracted and placed on a cooled Petri dish, where brains were isolated, and the hippocampi dissected. Samples were then transferred to a centrifuge tube and washed three times in Hank's Balanced Salt Solution (HBSS). The extracted tissue was treated with trypsin at 37 °C for 10 min,

washed three times in HBSS, and then mechanically dissociated using Pasteur pipettes. The NSCs were cultured in suspension in T75 flasks (Falcon) to obtain neurospheres, which were then collected, subjected to a centrifuge process, and mechanically dissociated to a single-cell suspension [62]. NSCs were cultured in suspension in DMEM/F-12 GlutaMAX (GIBCO, ThermoFisher Scientific, USA), 2% B27 supplement (GIBCO, ThermoFisher Scientific, USA), 1% N2 supplement (GIBCO, ThermoFisher Scientific, USA), 1% Pen/Strep (GIBCO, ThermoFisher Scientific, USA), 20ng/ml EGF (Peprotech, ThermoFisher Scientific, USA), and 20 ng/ml bFGF (Peprotech, ThermoFisher Scientific, USA). NSCs were transferred in a portable incubator (model CellTrans 2018, Labotect, Germany) from Verona to Modena and maintained in an incubator (37 °C, O₂/CO₂ 95%/5%) for 1 week before *in vivo* injections. Before transplantation, a subset of NSCs was used for immunofluorescence analysis to confirm the proper cell phenotype. NSCs were grown in adhesion for 24h, and the expression of markers related to cell proliferation (Ki67), stemness (SOX2, Nestin), intermediate neuronal progenitors (Doublecortin - DCX), and immature neurons (b3 tubulin - TUBB3) was assessed. As expected, most cells expressed the stemness markers SOX2 (69.78±6.80% positive cells over total nuclei) and Nestin (30.25±1.75% positive cells over total nuclei), while 28.89±1.73% of cells were proliferating Ki67+. Regarding the expression of markers related to the neuronal lineage, 28.43±1.75% of cells were neuronal progenitor cells (DCX+), and 7.29±1.11% of cells expressed the immature neuronal cell marker TUBB3. Overall, NSCs used for transplantation display mainly a neural stem cell phenotype.

Behavioural tests

The study of behavioural patterns was performed following a precise sequence: OF, NOL, EPM, social interaction test, and social behaviour in a resident-intruder paradigm. Behavioural performances were recorded by video-tracking and analysed using ANY-maze Software version 7.16. All tests were conducted in the light phase and completed within 16 days. Animals were handled, weighed, and moved to a clean cage once per week before starting the experiments to reduce the stress level during the behavioural experimental procedures. The animals were placed in the test room for a minimum of 30 min to 1 h prior to the testing procedure. All behavioural apparatuses were thoroughly cleaned between each rat performance using ethanol 70% to minimize bias due to olfactory cues [63]. All testing procedures were performed with the experimenters blinded to the rat group allocation; group identity was revealed only when statistical tests were performed.

Open Field (OF)

The OF test is commonly used to investigate locomotor activity and exploration [64], but it is also validated for the measurement of anxiety-related behaviour. The employed arena was 100x70 cm with a wall height of 38 cm. Animals were placed in the centre of the arena freely to move for 10 min. Total distance travelled was considered as an indicator of locomotor activity. Time spent in different arena subsegments was used for the estimation of anxiety level. Increased anxiety results in less locomotor motion and a preference for the edges (borders and corners) of the field. Other ethological parameters (i.e., climbing, rearing) were used to evaluate the patterns of exploratory behaviour [65] [66] [67] and emotional and motivational status of rats (i.e., grooming) [68]. The behaviours were observed and quantified manually by an observer who was unaware of the grouping of the subjects. The increased exploratory behaviour exhibited by the subjects suggests a concomitant decrease in anxiety levels.

Novel object location (NOL)

The NOL test investigates memory and spatial learning, which relies heavily on hippocampal activity [63]. The arena was the same as for the OF test. In the habituation phase, the animal is placed into the arena in the presence of two objects. In the testing phase (24 h after), one of the two objects is relocated to a new position. This test measures the spontaneous tendency of rodents to spend less time exploring a familiar object and to recognize when an object has been relocated. The success or the failure of the animal to complete the task has been identified by the discrimination index, calculated by the following formula: $(\text{Time}_{\text{NewObj}} - \text{Time}_{\text{OldObj}}) / (\text{Time}_{\text{NewObj}} + \text{Time}_{\text{OldObj}})$. Positive discrimination index values indicate good discrimination, since it means more time investigating the object in a new location. Discrimination index equal to zero indicates no preference, while a negative value could indicate neophobia to the novel object [63] [69].

Elevated Plus Maze (EPM)

The EPM test was used to evaluate anxiety [70]. The maze is made of a central platform (10x10 cm), two open and two closed arms (each 50x10 cm) 60 cm above the floor (Ugo Basile, Italy). The closed arms had 40 cm walls and an opaque cover. The animals were placed in the central square in front of an open arm and left free to explore the EPM platform for 5 min. The standard measures consisted of the percentage of time the animals spent exploring the open arm maze setting. Time spent in the closed arms and in the centre area was also evaluated.

Social interaction test

The social interaction test was performed to study social behaviour. The experimental room is composed of a Plexiglas cage (79x48 cm, height: 40 cm) with a net that separates the area into two different compartments. The first day, each animal (resident) was placed in the experimental arena for 10 min for habituation. The following day, the resident met the intruder for the first time. The tests lasted 10 min. The total time that the two animals spent interacting was measured. Once the test was completed, both resident and intruder animals were relocated to their respective home cages.

Resident-intruder paradigm

The resident-intruder paradigm was performed to study aggression and communication [71]. During the test, each animal (resident) was placed in the experimental Plexiglas cage (79x48 cm, height: 40 cm) for 30 min before meeting an unfamiliar untreated intruder and their social interaction was recorded for 5 min. Once the test was completed, both resident and intruder animals were relocated in their respective home cages. The time spent interacting or exhibiting aggressive behaviour was calculated, as well as the latency at first interaction or attack. The social behaviour was described into five different categories: communicative (pouncing, pinning, wrestling, body sniffing, and following), individual (moving on place, locomotion, freezing, auto-grooming, climbing, and rearing), sexual-like (mounting and genitalia sniffing), aggression and defence. The number of behaviours and the average time of each category were calculated. The graphs were used to illustrate the time spent engaging in a specific behaviour, as well as the probability of transitioning from one form of behaviour to another [65]. For the graph network visualization, Gephi software was used [72].

Perfusion and tissue processing

Rats were anesthetized with isoflurane and the abdomen was cut with a lateral incision, which continued along the rib cage on both sides. The heart was exposed, and the left ventricle was incised to facilitate the insertion of an olive-tipped perfusion needle into the ascending aorta. Additionally, the animal's right auricle was incised and saline solution containing CaCl_2 [2 mM] (150 ml, body temperature) was infused to remove all blood traces. Next, a solution of paraformaldehyde 4% and sucrose 4% in NaCl/Hepes/ CaCl_2 [135/20/2 mM] (approx. 200 ml, ice cold) was perfused through a peristaltic pump with a flow rate of 10 ml/min for 20 min. Brains were extracted and left for 24 h in a fixative solution at 4 °C. They were then transferred to a sterile sucrose solution, prepared with sucrose 30% in NaCl/Hepes/ CaCl_2 , and stored at 4 °C.

Subsequently, the brains were transferred to a temperature of -80 °C and coronally sliced (30 µm thickness) with a cryostat (Leica CM1520). Slices were stored at -20 °C in an anti-freezing cryoprotectant solution (glycerol 50% in NaCl/Hepes/CaCl₂) until the histological procedure. Animals were perfused at chronic timepoints, with a variability of 5-30 weeks post-IBO injection, as detailed in Tables S1 and S2. Beyond sham rats, where no significant differences are expected across different time points, the animals perfused 30 weeks post-injection correspond to non-epileptic rats that received 2 µl of IBO. This dose was soon abandoned in favour of the 0.3 µl dose, as it was not excessive while still being sufficient to ablate epileptic cells. Importantly, all rats included in the analysis of alginate effects on glial cells are relatively homogeneous with respect to the chronic post-injection interval, ranging from 9 to 10 weeks. Only 4 epileptic rats injected with ALG (n=1), NSCs (n=1), or both (n=2) were perfused at 5 weeks post-injection to verify injection accuracy and mid-term stem cell viability. Given that the analyses focused on a well-defined chronic immune response, this variability in perfusion timepoints is not expected to affect the results.

Histological procedures

Haematoxylin&Eosin (H&E)

After brain slices were left to dry onto glass slides, they were soaked in NaCl/Hepes/CaCl₂ [135/20/2 mM] for 1 min and then dipped in Mayer's Haematoxylin 0.1% (O.Kindler, Orsatec, Germany) for 3 min and rinsed in MilliQ water. Subsequently, slides were soaked in Eosin-solution 0.2% (O.Kindler, Orsatec, Germany) for 30 s, rinsed in MilliQ water, and dipped in ethanol at increasing concentrations: 50% (4 min), 70% (3 min), 90% (1.30 min), and 100% (1 dip). The slices were immersed in two distinct xylene solutions for a period of 5 and 25 min, respectively. Finally, they were mounted with Eukitt (Electron Microscopy Sciences, United States), covered with coverglasses, and left to dry overnight.

Toluidine Blue

After brain slices were left to dry onto glass slides, they were soaked in distilled water for 5 min and then in Toluidine Blue 0.1% solution (Carlo Erba Reagents, Italy) for 1 min. After a brief rinse in distilled water, slides were soaked in ethanol at increasing concentrations: 50% (30 s), 95% with 3 drops of acetic acid/100 ml (15 s), 100% (5 min). The slices were submerged in two distinct xylene solutions for a period of 5 min each and then mounted with Eukitt (Electron Microscopy Sciences, United States), covered with coverglasses, and left to dry overnight.

Immunofluorescence

Brain slices were washed in NaCl/Hepes/CaCl₂ [135/20/2 mM] solution (3 x 10 min, room temperature - RT). Samples were left in blocking solution (NaCl/Hepes/CaCl₂ [135/20/2 mM], Triton X 0.25%, bovine serum albumin - BSA 2%) with gentle shaking for 1 h at RT. Brain slices were then incubated overnight at 4 °C with anti-GFAP (rabbit, 1:2000, Dako, Z033429-2) or anti-Iba1 (rabbit, 1:1000, Wako, 019-19741) primary antibody in Triton solution (NaCl/Hepes/CaCl₂ [135/20/2 mM], Triton X-100 0.25%). The day after, sections were washed in Triton solution (3 x 10 min, RT), before incubation with donkey anti-rabbit Alexa Fluor 488 (donkey, 1:400, Thermo Fisher Scientific, A21206) in blocking solution for 3 h at RT. Following washes with Triton solution (3 x 10 min, RT) and with NaCl/Hepes/CaCl₂ (1 x 10 min, RT), brain slices were coverslipped with DAPI Fluoromount-G (Southern Biotech, 0100-20).

Immunohistochemistry

Slices were first washed in NaCl/Hepes/CaCl₂ solution (3 x 10 min, RT). Peroxidases were inhibited with H₂O₂ 3% in NaCl/Hepes/CaCl₂ solution (10 min, RT), then slides were washed again in NaCl/Hepes/CaCl₂ solution (3 x 10 min, RT). Pre-incubation blockage was carried out with a solution of BSA 0.1% in NaCl/Hepes/CaCl₂ [135/20/2 mM] in agitation for 1 h at RT. After, slices were incubated overnight at 4 °C with anti-Iba1 (rabbit, 1:1000, Wako, 019-19741) primary antibody, normal goat serum 1% (Vector Laboratories), and NaCl/Hepes/CaCl₂ 1X -Triton 0.3%. The next day, 3 sequential washings were made at RT: (i) NaCl/Hepes/CaCl₂ 1X -Triton 0.2%, (ii) NaCl/Hepes/CaCl₂ 1X -Triton 0.1%, (iii) NaCl/Hepes/CaCl₂ 1X. Then slices were incubated for 1 h at RT with a solution made by biotinylated anti-chicken IgG (1:300, Vector Laboratories, BA-9010) secondary antibody, normal goat serum 1%, and NaCl/Hepes/CaCl₂-Triton 0.2%. Next, two washings in NaCl/Hepes/CaCl₂ solution for 10 min at RT were carried out. Later, slides were incubated for 45 min in a solution of horseradish peroxidase streptavidin (1:300, Vector Laboratories, SA-5704), normal goat serum 1%, and NaCl/Hepes/CaCl₂ 1X (solution must be prepared 30 min before usage). A 10-min washing in NaCl/Hepes/CaCl₂ 1X was done before two washings with Tris-HCl 50 mM pH 7.4 for 10 min at RT. Slices were washed for 5 min with a solution made by 3,3'-diaminobenzidine (DAB) and Tris-HCl 50 mM (DAB/Tris-HCl 50 mM=0.001 g/2 ml). Immediately after, 100 µl of H₂O₂ 3% (in Tris) per 10 ml of DAB were added to the slices for 6-7 min. When slices were coloured, the reaction was blocked with Tris-HCl 50 mM and then washed 3 x NaCl/Hepes/CaCl₂ 1X. Then brain slices were soaked 5 min in the

following solutions: MilliQ water, ethanol 50%, ethanol 70%, ethanol 90%, ethanol 100%, xylene (twice). After, slides were mounted with Eukitt (Electron Microscopy Sciences; United States), covered with coverglasses, and left to dry overnight.

Imaging Measurements

Experimenters were blind to treatment group during image analysis. Fluorescence and brightfield images on brain slices (1-5 per injection; Table S1 and S2) were captured using the ECLIPSE Ci Microscope (Nikon Corporation, Japan; 4x, 10x, 20x images), ECLIPSE Ti2 Confocal Microscope (Nikon Corporation, Japan; 60x images) or EVOS M5000 (Thermo Fisher Scientific, USA; 100x images), and analysed offline with NIS-elements BR Software (Nikon Corporation, Japan). Firstly, the correct localization of multiple injections was visualized and confirmed by immunofluorescence (red mark for alginate, no signal for IBO-induced lesion); non-optimal injections characterized by misplacement of the injected materials were excluded from the analysis. In addition, the area of the vCA3 has been used for measurements, while regions above it, including the mechanical damage caused by both needle and cannula insertion, were carefully excluded; therefore, cannula and needle sizes did not affect the results.

The percentage of unlesioned CA3 out of the total length was determined on slices with AP coordinates between -5.28 and -5.76 mm stained with the H&E or Toluidine Blue.

Immune modulation was quantified by measuring GFAP and Iba1 IR mean intensity on the FITC channel [30] [73] [74] [75], excluding both TRITC (alginate) and DAPI channels. Specifically, we considered 15 regions of interest (ROIs), 8000 μm^2 in size, located in the perilesional area within a radius of approximately 500 μm (Supplementary materials, Fig. S3A). Acquisition parameters (exposure and gain) were kept constant across samples, and the data were normalized using an internal control of 15 ROIs of 8000 μm^2 in the perirhinal cortex, an area remote from the lesion. For qualitative visualization purposes only, in some experimental batches, the TRITC signal was overexposed to demonstrate the presence and correct localization of alginate. However, the analysis of Iba1 and GFAP mean intensity has been performed on the FITC channel only and using non-overexposed images.

To determine glial scar thickness, an average of 22 segments measuring the distance between the edge of the lesion and the edge of the reactive GFAP⁺ cells was considered for each slice [76] (Supplementary materials, Fig. S3B).

Iba1 cell body size, cell density, and morphological analyses were determined considering clearly defined nuclei within ROIs (1-3, 223 x 314 μm^2) located in the most reactive perilesional areas [77] (Supplementary materials, Fig. S3C). For the morphological analysis, the number of surveillance microglial cells, characterized by the presence of long, fine, and complex processes [78], was quantified and expressed relative to the total number of Iba1⁺ cells within each ROI.

Statistical analysis

Origin Pro 2024 (OriginLab, USA) software was routinely used for statistical analysis and graph plotting. The Kolmogorov-Smirnov normality test, or its refinement, the Anderson-Darling test, was used to assess the normal distribution of the data. Statistical significance was assessed by applying the following tests: (i) one-way ANOVA, followed by Tukey's post-hoc test for multiple comparisons, (ii) two-way ANOVA for repeated measures, and (iii) Kruskal-Wallis, followed by Dunn's test, when data were not normally distributed. Data were considered statistically significant at $p < 0.05$. Data are plotted as mean $\pm$ standard error of the means (SEM) or median [confidence interval 5-95]. "n" refers to the number of single injections in the histological analyses and to the number of rats in behavioural tests.

Data availability

[The datasets generated and analysed during the current study will be made publicly available in an open-access repository upon acceptance of the manuscript. The corresponding DOI will be provided at the proof stage.]

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Author contributions

Conceptualization FR, SB, ER and GC; formal analysis FR, SB, ER, BCG, AC, AV, SD; investigation FR, SB, ER, BCG, AC, GDR, AV, FC, SD, GP; funding acquisition ID, GP, GC; supervision MZ, ID, GP, GC; writing first draft FR, AV, GP, GC; all authors have read and agreed to the published version of the manuscript.

Additional information

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary information

[Supplementary material is uploaded separately]

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