Original article

Title: Cell encapsulation enhances antidepressant effect of the mesenchymal stem cells and counteracts depressive-like behavior of treatment-resistant depressed rats

Running title: Treatment effect of encapsulated MSCs

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ABSTRACT

Despite the advances in pharmacological therapies, only the half of depressed patients respond to currently available treatment. Thus, the need for further investigation and development of effective therapies, especially those designed for treatment-resistant depression, has been sorely needed. Although antidepressant effects of mesenchymal stem cells (MSCs) have been reported, the potential benefit of this cell therapy on treatment-resistant depression is unknown. Cell encapsulation may enhance the survival rate of grafted cells, but the therapeutic effects and mechanisms mediating encapsulation of MSCs remain unexplored. Here, we showed that encapsulation enhanced the antidepressant effects of MSCs by attenuating depressive-like behavior of Wistar Kyoto (WKY) rats, which are considered as a promising animal model of treatment-resistant depression. The implantation of encapsulated MSCs (eMSCs) into the lateral ventricle counteracted depressive-like behavior and enhanced the endogenous neurogenesis in the subventricular zone (SVZ) and the dentate gyrus (DG) of the hippocampus, whereas the implantation of MSCs without encapsulation or the implantation of eMSCs into the striatum did not show such ameliorative effects. eMSCs displayed robust and stable secretion of vascular endothelial growth factor (VEGF), brain derived neurotrophic factor, fibroblast growth factor-2, and ciliary neurotrophic factor (CNTF), and the implantation of eMSCs into the lateral ventricle activated relevant pathways associated with these growth factors. Additionally, eMSCs upregulated intrinsic expression of VEGF and CNTF and their receptors. This study suggests that the implantation of eMSCs into the lateral ventricle exerted antidepressant effects likely acting via neurogenic pathways, supporting their utility for depression treatment.

INTRODUCTION

Depression is one of the most common diseases and leading cause of disability around the world[1, 2]. Despite advances in pharmacological therapies, only half of depressed patients respond to currently available treatment[3]. Hence, there is an urgent unmet clinical need for further investigation and development of effective therapies, especially for treatment-resistant depression[4]. Treatment-resistant depression is associated with risk factors, such as congenital vulnerability to depression and resistance to conventional antidepressants[5]. In preclinical studies, Wistar Kyoto (WKY) rats exhibit high level of depression-like behavior congenitally[6-9] and show resistance to conventional antidepressant treatment[10-12]. Accordingly, WKY rats stand as a promising model of treatment-resistant depression[4].

Mesenchymal stem cells (MSCs) pose as effective candidates for cell therapy in treating neurodegenerative diseases, such as Parkinson's disease[13], multiple sclerosis[14], and stroke[15]. The therapeutic effects of MSC implantation have been explored in depression. Intrahippocampal implantation of MSCs promote neuroplasticity but does not produce antidepressant effects in Lewis rats[16]. However, intraventricular implantation of MSCs exert antidepressant effects in Flinders sensitive line (FSL) rats[17], another animal model for depression[18]. To date, it is unknown whether cell-to-cell contact between transplants and host cells, or transplanted cell-secreted factors mediate the observed beneficial effects of MSCs.

Encapsulation of various cell lines has revealed secretion of neurotrophic factors may underlie the therapeutic action of stem cells in certain target diseases[19-21]. Transplanted MSCs may confer their functional benefits via secretion of neurotrophic factors rather than MSCs graft survival and integration with the host per se[22]. The feasibility of encapsulated MSCs (eMSCs) in affording antidepressant effects remained unexplored until now. In this study, we examined the functional effects and mechanism of action associated with eMSCs in a treatment-resistant depression animal model.

MATERIALS AND METHODS

Animals

Male WKY rats (8 weeks years old at the beginning of the experiment; Charles River, Yokohama, Japan) were used as subjects in this experiment. Rats were group-housed with two animals per cage within strain. The animal housing facility was maintained on 12h light/dark cycle, and the animals had free access to food and water. Animals were excluded from this study only if there was excessive damage caused by the surgical procedure (e.g., uncontrollable bleeding). All analyses were performed blinded.

All testing procedures were approved by the Institutional Animal Care and Use Committee of Okayama University (protocol #OKU- 2017045).

Bone marrow-derived MSCs isolation and expansion

MSCs were isolated from the bone marrow of male Wistar rats (6 weeks years old; Charles River) as previously described[23]. See Supplementary Materials and Methods for detailed procedures.

Encapsulation of MSCs

Polymeric hollow fibers (15cm in length, 500 μm in cross-sectional inner diameter) consisting of a semipermeable membrane (Amicon, Beverly, MA, United States) were cut to 7mm in length and used as

capsules as previously described[19]. These fibers were made of polysulfone (molecular cut-off: 100 kDa). MSCs were prepared as single-cell suspension at a density of 6×10^4 cells/µl and 5µl was loaded. After 1 week in culture, we checked if MSCs leaked out from the capsule as a quality control procedure. Capsules from which MSCs leaked out were not used for implantation. Thereafter, eMSCs were implanted. Empty capsules were made in the same way except that cell loading was not performed. Some eMSCs were used for Hematoxylin and Eosin staining to reveal viable MSCs inside the capsule. See Supplementary Materials and Methods for detailed procedures.

Surgical procedures

All surgical procedures were performed on day 0. Under general anesthesia (1% halothane in 69% N_2O and 30% O_2), MSCs (3×10^5 cells/5µl), eMSCs, or an empty capsule was implanted stereotaxically into the right striatum or the right lateral ventricle. One capsule was implanted to one rat.

See Supplementary Materials and Methods for detailed procedures.

Behavioral tests (open field test and forced swim test)

Seventy rats were randomly assigned to 7 groups for behavior tests (control group, MSCs into the lateral ventricle (MSCs-LV) group, eMSCs into the lateral ventricle (eMSCs-LV) group, empty capsule into the lateral ventricle (EC-LV) group, MSCs into the striatum (MSCs-ST) group, eMSCs into the striatum group (eMSCs-ST), and empty capsule into the striatum (EC-ST) group; n = 10 per group). Rats in the control group did not receive any surgical procedure. The other groups of rats received surgical procedures as described in “Surgical procedures” section.

All behavioral tests were performed and analyzed using Ethovision® XT 9.0 software (Noldus, Wageningen, Netherlands) as previously described[6]. The experimental setup used in the behavior groups is presented in Figure 1a. In the OFT, the total distance rats walked was measured. In the FST, the percentage of time rats spent immobile was measured.

To show the reference data, the same behavioral tests were conducted in Wistar rats as a healthy control group and WKY rats treated with Fluoxetine (Tokyo chemical industry co., Tokyo, Japan) as a conventional treatment group (n = 10 per group).

See Supplementary Materials and Methods for detailed procedures.

Immunohistochemistry and cell counting

A separate cohort of ten rats were assigned to eMSCs-LV treatment without behavioral tests (eMSCs-LV w/o BT) to delineate the true functional benefits of encapsulation from potential confounding effects of behavioral tests on neurogenesis.

All rats from 7 groups subjected to behavioral tests as well as those rats included in eMSCs-LV w/o BT group were processed for immunohistochemistry. To evaluate the endogenous neurogenesis in the subventricular zone (SVZ) and the dentate gyrus (DG) of the hippocampus, 5-bromo-2'-deoxyuridine (BrdU, NACALAI TESQUE INC., Kyoto, Japan) was administered to all rats at a concentration of 50 mg/kg body weight, with five consecutive intraperitoneal injections every 12h from day 13 to 15. At the end of this BrdU protocol, rats were sacrificed and perfused, their brains removed and postfixed. Some eMSCs were explanted from the rats in the eMSCs-LV group and used for Hematoxylin and Eosin staining to demonstrate

surviving MSCs inside the capsule. BrdU/Doublecortin (Dcx) double staining and quantification of BrdU and BrdU/Dcx positive cells in the SVZ and DG were performed as previously described[6, 24, 25]. The antibodies included anti-BrdU antibody (1:100, OBT0030G; Bio-Rad, Hercules, California, United States), rabbit anti-Dcx antibody (1:200, #4604; Cell Signaling Technology, Danvers, Massachusetts, United States), biotinylated anti-rat secondary antibody (1:100, 712-065-153; Jackson Immunoresearch, West Grove, Pennsylvania, United States), strepatavidin Alexa-488 (1:200, S11223; Invitrogen, Carlsbad, California, United States), goat anti-rabbit Cy3 (1:200, ab97075; abcam, Cambridge, United Kingdom), and 4,6-diamidino-2-phenylindole (DAPI; 1:500, D3751; Thermo Fisher). See Supplementary Materials and Methods for detailed procedures.

Enzyme-linked immunosorbent assay (ELISA)

Another cohort of 40 rats were randomly assigned to 5 treatment groups for processing with ELISA (control group, MSCs-LV group, eMSCs-LV group, MSCs-ST group, eMSCs-ST group; n = 8 per group). Behavioral tests were not conducted for this cohort. All groups of rats except for the control group received MSCs or eMSCs implantation (day 0). Encapsulation of MSCs and surgical procedures were performed in the same method described above in "Encapsulation of MSCs" and "Surgical procedure". After 1 week in culture, the supernatant of eMSCs was collected to evaluate levels of neurotrophic factors released at the pre-implant eMSCs. Thereafter, eMSCs were used for surgical procedure.

On day 15, under deep anesthesia with pentobarbital (100 mg/kg), bilateral hippocampi of rats was dissected on ice. The capsule was explanted from the rats of the eMSCs-LV group at the same time and used for the collection of supernatant of the post-explant eMSCs.

Levels of vascular endothelial growth factor (VEGF), brain derived neurotrophic factor (BDNF), fibroblast growth factor-2 (FGF-2), and ciliary neurotrophic factor (CNTF) in the hippocampus and supernatant of the pre-implant and post-explant eMSCs were determined using ELISA kit (VEGF: Quantikine RRV00; R&D Systems, Minneapolis, Minnesota, United States, BDNF: Quantikine DBNT00; R&D Systems, FGF-2: Quantikine MFB00; R&D Systems, CNTF: ab100758; abcam). See Supplementary Materials and Methods for detailed procedures.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

To evaluate the mRNA expression of VEGF, BDNF, FGF-2, and CNTF in the hippocampus, another cohort of 30 rats were randomly assigned to 5 treatment groups for processing with qRT-PCR (control group, MSCs-LV group, eMSCs-LV group, MSCs-ST group, eMSCs-ST group; n = 6 per group). Total RNA was isolated from bilateral hippocampi, and synthesized cDNA with the SuperScript III First-Strand Synthesis System (Invitrogen). The qRT-PCR reactions were then performed and detected using the StepOnePlus system (Life Technologies, Carlsbad, CA, USA). As an internal control, we used GAPDH mRNA. Primers were purchased from Invitrogen. See Supplementary Materials and Methods for detailed procedures.

Western immunoblotting using brain homogenates

A new cohort of 50 rats were randomly assigned to 5 treatment groups for processing with western immunoblotting (control group, MSCs-LV group, eMSCs-LV group, MSCs-ST group, eMSCs-ST group; n = 10 per group). Behavioral tests were not conducted for this cohort. The hippocampal expression of VEGF receptor 2 (VEGFR2), phosphorylated VEGFR2 (pVEGFR2), tropomyosin receptor kinase B (TrkB),

phosphorylated TrkB (pTrkB), fibroblast growth factor receptor 1 (FGFR1), phosphorylated FGFR1 (pFGFR1), CNTF receptor alpha (CNTFR alpha), Signal Transducers and Activator of Transcription 3 (STAT3), and phosphorylated STAT3 (pSTAT3) was evaluated.

The antibodies included anti-VEGFR2 (1:1000, ab39256; abcam), anti-pVEGFR2 (1:1000, ab5473; abcam), anti-TrkB (1:1000, ab18987; abcam), anti-pTrkB (1:1000, ab109684; abcam), anti-FGFR1 (1:500, ab58516; abcam), anti-pFGFR1 (1:500, ab 59194; abcam), anti-CNTFR alpha antibody (1:500, ab127425; abcam), anti-STAT3 antibody (1:1000, #12640; Cell Signaling Technology), anti-pSTAT3 antibody (1:2000, #9145; Cell Signaling Technology), and ß-actin (1:5000, A5441; Sigma-Aldrich), with anti-mouse and anti-rabbit IgG HRP-linked secondary antibodies (both 1:5000, Cell Signaling Technology). See Supplementary Materials and Methods for detailed procedures.

Labeled MSCs implantation to the lateral ventricle

To evaluate the existence of MSCs-LV, labeled MSCs were implanted to eight newly assigned rats. MSCs were labeled with a Q-Tracker 625 Cell Labeling kit (A10198, Invitrogen) as previously described[26], and were injected to the lateral ventricle. At 3 days after implantation and 7 days after implantation, rats were sacrificed, and frozen coronal sections were evaluated (n = 4, each). See Supplementary Materials and Methods for detailed procedures.

Statistical analyses

All data were analyzed using SPSS ver. 20.0 software (SPSS, Chicago, Illinois, United States). Statistical significance was assessed by two-tailed *t*-test for comparisons between two groups. Statistical significance between multiple groups were analyzed by one-way analysis of variance (ANOVA) with subsequent Turkey's tests. Statistical significance was preset at $p<0.05$. The variance homogeneity was confirmed with Levene's test. Mean values are presented with standard error (SE). No statistical methods were used to predetermine sample sizes. Sample sizes are estimated based on previous publications.

RESULTS

Implantation of eMSCs-LV results in a behavioral change

Only rats treated with eMSCs-LV displayed a significantly long distance of movement in the OFT compared with the other 6 groups (Figure 1b). Additionally, except for day 1 (Figure 1c), rats of the eMSCs-LV group also exhibited a significant reduction in their immobility time in the FST day 2 compared with the other 6 groups (Figure 1d).

Implantation of eMSCs-LV increased the number of BrdU- and BrdU/Dcx-positive cells in the SVZ and the DG

The behavioral change was accompanied by neurogenic activity characterized by a significant increase in the number of BrdU- and BrdU/Dcx-positive cells in the SVZ of the eMSCs-LV group and eMSCs-LV w/o BT group compared to all other groups (Figure 2a, c, d). In addition to the neurogenic SVZ, the other neurogenic niche DG similarly displayed enhanced activity as detected in significant increase in the number of BrdU- and BrdU/Dcx-positive cells in the DG of the eMSCs-LV group and eMSCs-LV w/o BT group

compared with all other groups (Figure 2b, e, f).

There was no statistical significance between eMSC-LV group and eMSCs-LV w/o BT group in terms of the number of BrdU- and BrdU/Dcx-positive cells in the SVZ and the DG (P=1.0 for BrdU-positive cells in the SVZ, P=1.0 for BrdU/Dcx-positive cells in the SVZ, P=1.0 for BrdU-positive cells in the DG, P=1.0 for BrdU/Dcx-positive cells in the DG), suggesting that enhanced neurogenesis is a function of eMSC implantation and that the exposure of animals to behavioral tests in this study did not significantly affect the observed neurogenic activity.

MSCs survive encapsulation and eMSCs continued to secrete neurotropic factors

Hematoxylin and Eosin staining of the capsules retrieved at 15 days after transplantation demonstrated surviving MSCs inside the capsules, although the number of MSCs inside the retrieved capsules decreased compared to MSCs inside the capsule before implantation (Figure 3a, b).

Supernatant of the pre-implant and post-explant eMSCs implanted to rats for ELISA was used to evaluate the change of secretion of neurotrophic factors from eMSCs. The secretion of VEGF, BDNF, and CNTF from post-explant eMSCs did not show a significant decrease compared to those of pre-implant eMSCs, although trends of lower expression levels were observed (Figure 3c, d, f). On the other hand, FGF-2 secretion from the post-explant eMSCs were significantly lower compared to that from the pre-implant eMSCs (Figure 3e).

Implantation of eMSC-LV enhances the expression of endogenous VEGF and CNTF in the hippocampus

Coupled with the robust secretion of trophic factors by transplanted eMSCs, upregulated endogenous levels of specific trophic factors were also observed. The protein and mRNA expression levels of VEGF and CNTF were upregulated in the eMSCs-LV group compared to the other groups (Figure 4a, d, e, h). On the other hand, the protein and mRNA expression levels of BDNF and FGF-2 did not show any detectable upregulation (Figure 4b, c, f, g).

Implantation of eMSCs-LV activates multiple signaling pathways and enhances the expression of receptors for neurotrophic factors

In further support of a trophic factor-mediated mechanism underlying functional effects of eMSC transplanation, the expression of VEGFR2 and pVEGFR2 were upregulated in eMSCs-LV group compared to other groups (Figure 5a, b, c). Moreover, although the expression of TrkB did not change (Figure 5a, d), the expression of pTrkB was upregulated in eMSCs-LV group compared to other groups (Figure 5a, e). In the same fashion, while the expression of FGFR1 was not changed (Figure 5a, f), the expression of pFGFR1 was upregulated in eMSCs-LV group compared to other groups (Figure 5a, g). Finally, the expression of CNTFR alpha, STAT3, and pSTAT3 was upregulated in eMSCs-LV group compared to other groups (Figure 5a, h, i, j).

No MSCs were observed at 7 days after implantation

Although some labeled MSCs can be detected in the lateral ventricle on day 3 (Supplementary Figure S2), labeled MSCs existed neither in the lateral ventricle nor in the brain parenchyma at 7 days after implantation.

DISCUSSION

This study demonstrated for the first time that encapsulation enhanced the treatment effects of MSCs in an animal model of treatment-resistant depression. MSCs-LV were initially reported to show an antidepressant effect and promote neurogenesis in a rat model of depression[17]. Although we implanted more MSCs than this previous study[17], our study showed that MSCs-LV neither result in any behavioral change nor enhance neurogenesis in this treatment-resistant depression model. In contrast, eMSCs-LV show behavioral change and enhanced neurogenesis in treatment-resistant depressed rats. These findings suggest that encapsulation of MSCs stands as an improved therapeutic approach in promoting treatment effects when using the stringent treatment-resistant depression model, which likely represents a much more advanced pathological manifestation of depression. However, the treatment effect of eMSC-LV is different from the typical antidepressant profile. Conventional antidepressant drugs increase neurogenesis only in the DG[27]. A typical antidepressant profile is improvement in FST without the change in OFT. Therefore, our behavioral results are difficult to interpret. The obscurity of the pathology of WKY rats makes this problem more complicated. Further investigation with other depression animal models or behavioral tests may provide additional information.

We detected MSCs neither in the lateral ventricle nor in the brain parenchyma at 7 days after implantation into the lateral ventricle. Consistent with our results, MSCs implanted into the lateral ventricle of the rodent model of neurodegenerative disease display low engraftment even when assessed within only a few days post-transplantation[28-30]. Accordingly, the survival rate and engraftment of MSCs implanted into the lateral ventricle is deemed modest at best. Polymer encapsulation may be able to protect the grafted cells from immune system attack[31]. In this study, we detected the survival of MSCs in the capsule up to at least day 15 post-transplantation. Additionally, we showed that neurotrophic factors (VEGF, BDNF, FGF-2, and CNTF) were secreted from post-explant eMSCs. The polymer encapsulation likely increased the survival rate of MSCs, which in turn facilitated the eMSCs to maintain robust secretion of neurotrophic factors. Altogether, such encapsulation promoted the enhancement of antidepressant effect and positive modulation of neurogenesis. Despite these positive outcomes, the long-term effects of encapsulated MSCs is warranted in view of trends of decreased survival in the capsule and lower secretion of neurotrophic factors over time. As depression treatment can be a multi-year therapy, long-term treatment effects should be evaluated.

This study implicated the lateral ventricle as a more appropriate site of implantation than the striatum. The secretome of MSCs appears to play a major role in the therapeutic effect of MSCs[22]. With this in mind, the secretome from eMSCs-LV likely reached various sites of brain more easily than the secretome from eMSCs-ST. The pathology of depression remains incompletely understood and multiple culprit lesions, even remote from the lateral ventricle, may manifest the disease aberrations[32]. Delivering the secretome to multiple brain regions may be important to recognize therapeutic effects on depression.

Enhancing the secretome rather than facilitating survival and engraftment of MSCs may be the key mechanism in promoting antidepressant effects. MSCs implanted to the lateral ventricle migrate to the ipsilateral hippocampus in a rat model of depression [17, 33], but direct intrahippocampal transplantation of MSCs does not afford an antidepressant effect[16]. In the present study, transplanting eMSCs into the lateral ventricle reduced depressive-like behavior, suggesting that neither migration nor engraftment of MSCs in the hippocampus is necessary to achieve the therapeutic effect on depression.

In an effort to test the hypothesis that a trophic factor mechanism mediated the antidepressant

effects of eMSCs, we evaluated phosphorylation of VEGFR2, TrkB and FGFR1, which are receptors for VEGF, BDNF, and FGF-2 respectively. The results revealed that eMSCs robustly secrete VEGF, BDNF, and FGF-2, and the expression levels of phosphorylated receptors for these factors was upregulated by eMSCs-LV. These findings suggest that eMSCs-LV activated the pathway of VEGF, BDNF, and FGF2, which are factors associated with pathologic manifestation of depression[32, 34-39], and interestingly these same factor may serve as therapeutic targets for modulating neurogenesis[40-42]. Indeed, mono-infusion of any these factors either into the DG[43] or lateral ventricle[40, 44] exerts antidepressant effect.

To further clarify the interaction between neurogenesis and depression, we focused on CNTF, which was reported to modulate anxiety and depressive-like behavior[45] and is suggested to contribute to neurogenesis[46, 47]. To evaluate the activation of CNTF pathway, we evaluated the expression of STAT3 and pSTAT3. STAT3 is a known downstream product of CNTF signaling, with STAT3 phosphorylated by activation of CNTF pathway[48]. Here, we observed that eMSCs secreted CNTF and the expression of pSTAT3 was upregulated, suggesting that CNTF pathway was activated by eMSCs-LV. It was previously found that CNTF secreting capsule implanted into the lateral ventricle improved motor skills of aged rats[49]. The behavioral change of eMSC-LV may be psychomotor improvement[50] that may involve the CNTF pathway as one of the mechanism. To this end, the Jak-STAT pathway acting as positive autoregulatory loop[51, 52] of STAT3 may correspond to one of the multiple signaling pathways mediating the neurogenic modulation of depression.

Along this line of neurogenic role in depression, eMSCs-LV enhanced the expression of VEGF, VEGFR2, CNTF, and CNTFR alpha (a receptor for CNTF). MSCs implantation upregulates the expression of VEGF in a stroke rat model[53, 54]. Intrahippocampal implantation of dental pulp stem cells, which share some phenotypic features with MSCs, enhances the endogenous expression of VEGF and CNTF[55], highlighting the concept that nurturing the microenvironment of the brain may participate in producing an antidepressant effect. CNTF behaves in an autocrine manner[56], whereas VEGFR2 plays essential roles in neurogenesis[57, 58], further suggesting a close interaction between depressive-like behavior and neurogenesis[45, 46] in conferring an antidepressant effect.

As limitation, it is unclear the treatment effect of eMSCs-LV based on which of the following: neurogenesis, the neurotrophic factors, or both of them. For example, experiments using a rat exposed to irradiation of the hippocampus or direct injection of neurotrophic factors may clarify this point. Additionally, it would be important to evaluate the treatment effect of intraventricular injection of such neurotrophic factors. If it ameliorates depressive behavior, our results would be supported and direct injection of neurotrophic factors can be a new treatment choice.

In summary, eMSCs-LV promoted antidepressant effect against the treatment-resistant depression model. Such encapsulation of MSCs offers a method for continuous supply of a cocktail of exogenous neurotrophic factors and upregulating intrinsic expression of neurotrophic factors and their receptors, altogether facilitating neurogenesis as a new treatment strategy for depression[59, 60].

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary information is available at MP's website.

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Figure 1. Intraventricular implantation of eMSCs counteracted depressive like behavior. (a) Experimental setup: Rats received surgical procedure (implantation of MSCs, eMSCs, or an empty capsule) on day 0. The OFT was performed on day 13, and the FST was performed on day 14 and 15. BrdU was administered intraperitoneally every 12 h on days 13 to 15 at a concentration of 50mg/kg. (b) Rats of eMSCs-LV group walked a significantly longer distance in the OFT compared with all other groups ($F_{(6,63)} = 5.675$, $^*P<0.05$ versus all other groups). (c) There was no significant immobility difference in the FST day 1. ($F_{(6,63)} = 1.529$, $P=0.17$). (d) Rats of eMSCs-LV group spent a significant lower percentage of time immobile than rats of all other groups in the FST day 2 ($F_{(6,63)} = 4.719$, $^*P<0.05$ versus all other groups).
The results of WKY rats treated with Fluoxetine and Wistar rats are shown as reference. $n = 10$ per group.
eMSCs, encapsulated mesenchymal stem cells; MSCs, mesenchymal stem cells; OFT, open field test; FST, forced swim test; BrdU, 5-bromo-2'-deoxyuridine; eMSCs-LV, eMSCs into the lateral ventricle; MSCs-LV, MSCs into the lateral ventricle; EC-LV, empty capsule into the lateral ventricle; MSCs-ST, MSCs into the striatum; eMSCs-ST, eMSCs into the striatum group; EC-ST, empty capsule into the striatum.

Figure 2. Intraventricular implantation of eMSCs increased neurogenesis in the SVZ and the DG. (a, b) Immunostaining for DAPI (blue), BrdU (green), and Dcx (red) shows that there is an increase in proliferation and neurogenesis in the SVZ and DG. (c) Quantification of the number of BrdU positive cells in the SVZ indicated that there is significant increase in the number of BrdU positive cells in rats of the eMSCs-LV group and eMSCs-LV w/o BT group ($F_{(7,72)} = 5.364$, $^*P<0.05$ versus control, MSCs-LV, EC-LV, MSCs-ST, eMSCs-ST, and EC-ST group). (d) There is a significant increase in the number of BrdU/Dcx positive cells in the SVZ of rats of the eMSCs-LV group and eMSCs-LV w/o BT group ($F_{(7,72)} = 4.331$, $^*P<0.05$ versus control, MSCs-LV, EC-LV, MSCs-ST, eMSCs-ST, and EC-ST group). (e) The number of BrdU positive cells in the DG significantly increased in rats of the eMSCs-LV group and eMSCs-LV w/o BT group ($F_{(7,72)} = 19.468$, $^*P<0.05$ versus control, MSCs-LV, EC-LV, MSCs-ST, eMSCs-ST, and EC-ST group). (f) There is a significant increase in the number of BrdU/Dcx positive cells in the DG of rats of the eMSCs-LV group and eMSCs-LV w/o BT group ($F_{(7,72)} = 12.986$, $^*P<0.05$ versus control, MSCs-LV, EC-LV, MSCs-ST, eMSCs-ST, and EC-ST group).
$n = 10$ per group.

eMSCs, encapsulated mesenchymal stem cells; SVZ, subventricular zone; DG, dentate gyrus; DAPI, 4,6-diamidino-2-phenylindole; BrdU, 5-bromo-2'-deoxyuridine; Dcx, Doublecortin; eMSCs-LV, eMSCs into the lateral ventricle; eMSCs-LV w/o BT, eMSCs-LV without behavior tests; MSCs-LV, MSCs into the lateral ventricle; EC-LV, empty capsule into the lateral ventricle; MSCs-ST, MSCs into the striatum; eMSCs-ST, eMSCs into the striatum; EC-ST, empty capsule into the striatum.

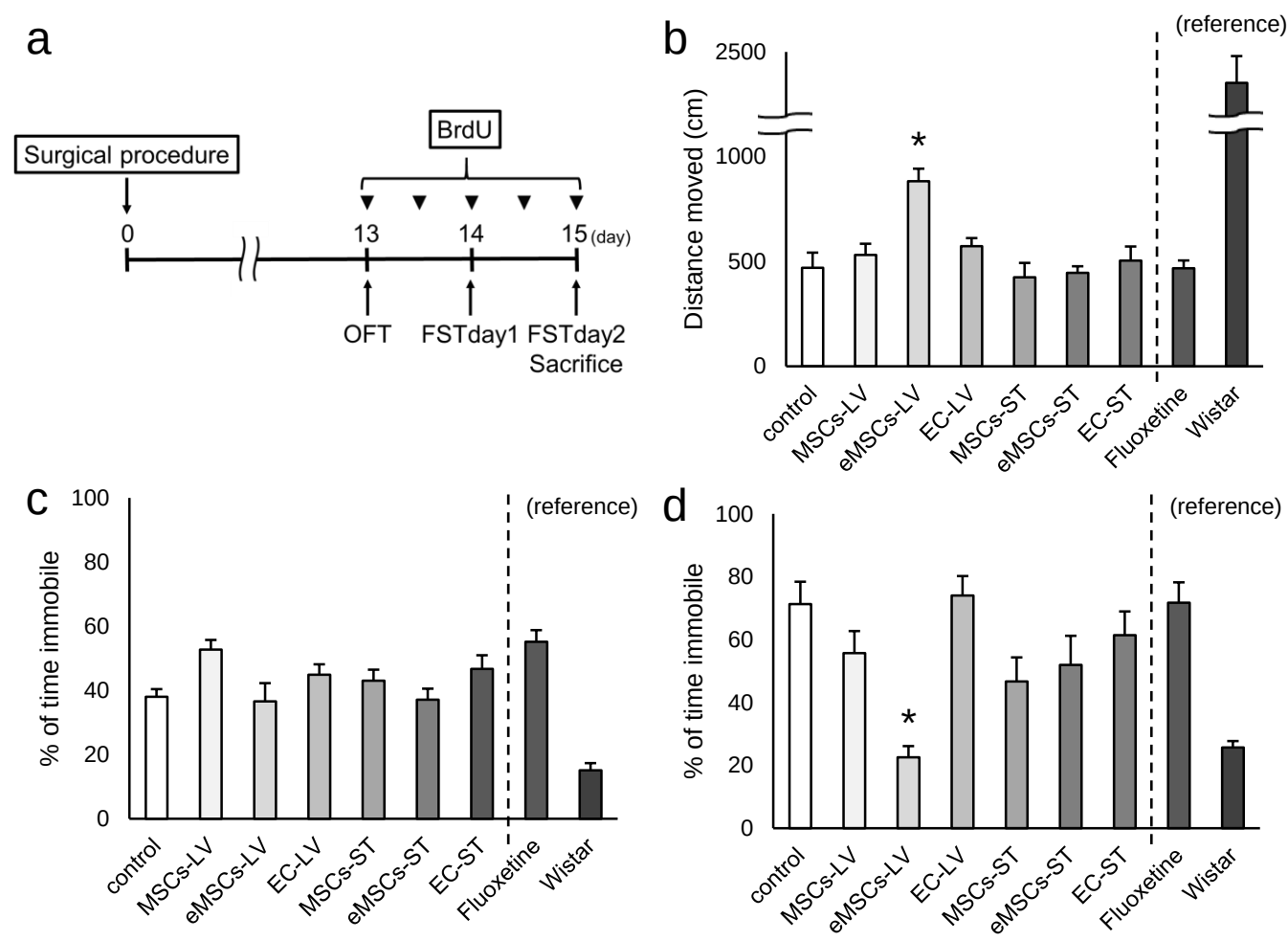
Figure 3. Neurotrophic factors were secreted from both of pre-implant and post-explant eMSCs. (a) Hematoxylin and Eosin staining of pre-implant eMSC. MSCs can be observed inside the capsule. (b) Hematoxylin and Eosin staining of post-explant eMSC. MSCs can be still observed inside the capsule, although the number of MSCs inside the post-explant capsule decreased compared to that of MSCs inside the pre-implant capsule. (c) No significant difference was found between the secretion of VEGF from pre-implant eMSCs and that of post-explant eMSCs, although the downward trends were observed (P=0.23). (d) Significant difference was also not found between the secretion of BDNF from pre-implant eMSCs and that of post-explant eMSCs, although the downward trends were observed (P=0.19). (e) The post-explant eMSCs secreted significantly lower FGF-2 compared to that from the pre-implant eMSCs (*P<0.05). (f) No significant difference was found between the secretion of CNTF from pre-implant eMSCs and that of post-explant eMSCs, although the downward trends were observed (P=0.05).
n = 8 per group.
eMSCs, encapsulated mesenchymal stem cells; MSCs mesenchymal stem cells; VEGF, vascular endothelial growth factor; BDNF, brain derived neurotrophic factor; FGF-2, fibroblast growth factor-2; CNTF, ciliary neurotrophic factor.

Figure 4. The protein and mRNA expression of VEGF and CNTF was upregulated in the hippocampus of rats of eMSCs-LV group. (a) Rats of eMSCs-LV group showed significantly high protein expression of VEGF in the hippocampus compared to all other groups ($F_{(4,35)}$ = 3.99, *P<0.05 versus all other groups). (b) There was no significant difference about the protein expression of BDNF ($F_{(4,35)}$ = 1.29, P=0.29). (c) There was also no significant difference about the protein expression of FGF-2 ($F_{(4,35)}$ = 0.724, P=0.581). (d) Rats of eMSCs-LV group showed significantly high protein expression of CNTF in the hippocampus compared to all other groups ($F_{(4,35)}$ = 6.20, *P<0.05 versus all other groups). (e) Rats of eMSCs-LV group showed significantly high mRNA expression of VEGF in the hippocampus compared to all other groups ($F_{(4,25)}$ =11.566, *P<0.05 versus all other groups). (f) There was no significant difference about the mRNA expression of BDNF ($F_{(4,25)}$ = 0.977, P=0.438). (g) There was also no significant difference about the mRNA expression of FGF-2 ($F_{(4,25)}$ = 1.135, P=0.363). (h) Rats of eMSCs-LV group showed significantly high protein expression of CNTF in the hippocampus compared to all other groups ($F_{(4,25)}$ = 17.484, *P<0.05 versus all other groups).
(a)-(d): n = 8 per group. (e)-(h): n = 6 per group.
VEGF, vascular endothelial growth factor; CNTF, ciliary neurotrophic factor; eMSCs-LV, encapsulated mesenchymal stem cells into the lateral ventricle; MSCs-LV, mesenchymal stem cells into the lateral ventricle; EC-LV, empty capsule into the lateral ventricle; MSCs-ST, mesenchymal stem cells into the striatum; eMSCs-ST, encapsulated mesenchymal stem cells into the striatum; EC-ST, empty capsule into the striatum; BDNF, brain derived neurotrophic factor; FGF-2, fibroblast growth factor-2.

Figure 5. Implantation of eMSCs-LV activated multiple signaling pathway and enhanced the expression of receptors for neurotrophic factors. (a) Western blots of hippocampus show that the expression of VEGFR2, pVEGFR2, pTrkB, pFGFR1, CNTFR alpha, STAT3, and pSTAT3 was upregulated. Protein levels were normalized to ß-actine. (b) Quantification of western blots by densitometric analysis indicated that the expression of VEGFR2 was upregulated ($F_{(4,45)}$ = 26.46, **P*<0.05 versus all other groups). (c) The expression of pVEGFR2 was also upregulated ($F_{(4,45)}$ = 5.10, **P*<0.05 versus all other groups). (d) The expression of TrkB was not changed ($F_{(4,45)}$ = 0.66, *P*=0.62). (e) The expression of pTrkB was upregulated ($F_{(4,45)}$ = 3.91, **P*<0.05 versus all other groups). (f) The expression of FGFR1 was not changed ($F_{(4,45)}$ = 1.97, *P*=0.12). (g) The expression of pFGFR1 was upregulated ($F_{(4,45)}$ = 4.12, **P*<0.05 versus all other groups). (h) The expression of CNTFR alpha was also upregulated ($F_{(4,45)}$ = 5.85, **P*<0.05 versus all other groups). (i) The expression of STAT3 was also upregulated ($F_{(4,45)}$ = 4.15, **P*<0.05 versus all other groups). (j) The expression of pSTAT3 was also upregulated ($F_{(4,45)}$ = 4.78, **P*<0.05 versus all other groups).

n = 8 per group.

eMSCs-LV, encapsulated mesenchymal stem cells into the lateral ventricle; VEGFR2, vascular endothelial growth factor receptor 2; pVEGFR2, phosphorylated VEGFR2; pTrkB, phosphorylated tropomyosin receptor kinase B; pFGFR1, fibroblast growth factor receptor 1; CNTFR alpha, ciliary neurotrophic factor receptor alpha; STAT3, Signal Transducers and Activator of Transcription 3; pSTAT3, phosphorylated STAT3; MSCs-LV, mesenchymal stem cells into the lateral ventricle; MSCs-ST, mesenchymal stem cells into the striatum; eMSCs-ST, encapsulated mesenchymal stem cells into the striatum; TrkB, tropomyosin receptor kinase B.


a
Surgical procedure
BrdU
0
13
14
15 (day)
OFT
FSTday1
FSTday2
Sacrifice
b
(reference)
Distance moved (cm)
2500
1000
500
0
*
control
MSCs-LV
eMSCs-LV
EC-LV
MSCs-ST
eMSCs-ST
EC-ST
Fluoxetine
Wistar
c
(reference)
% of time immobile
100
80
60
40
20
0
control
MSCs-LV
eMSCs-LV
EC-LV
MSCs-ST
eMSCs-ST
EC-ST
Fluoxetine
Wistar
d
(reference)
% of time immobile
100
80
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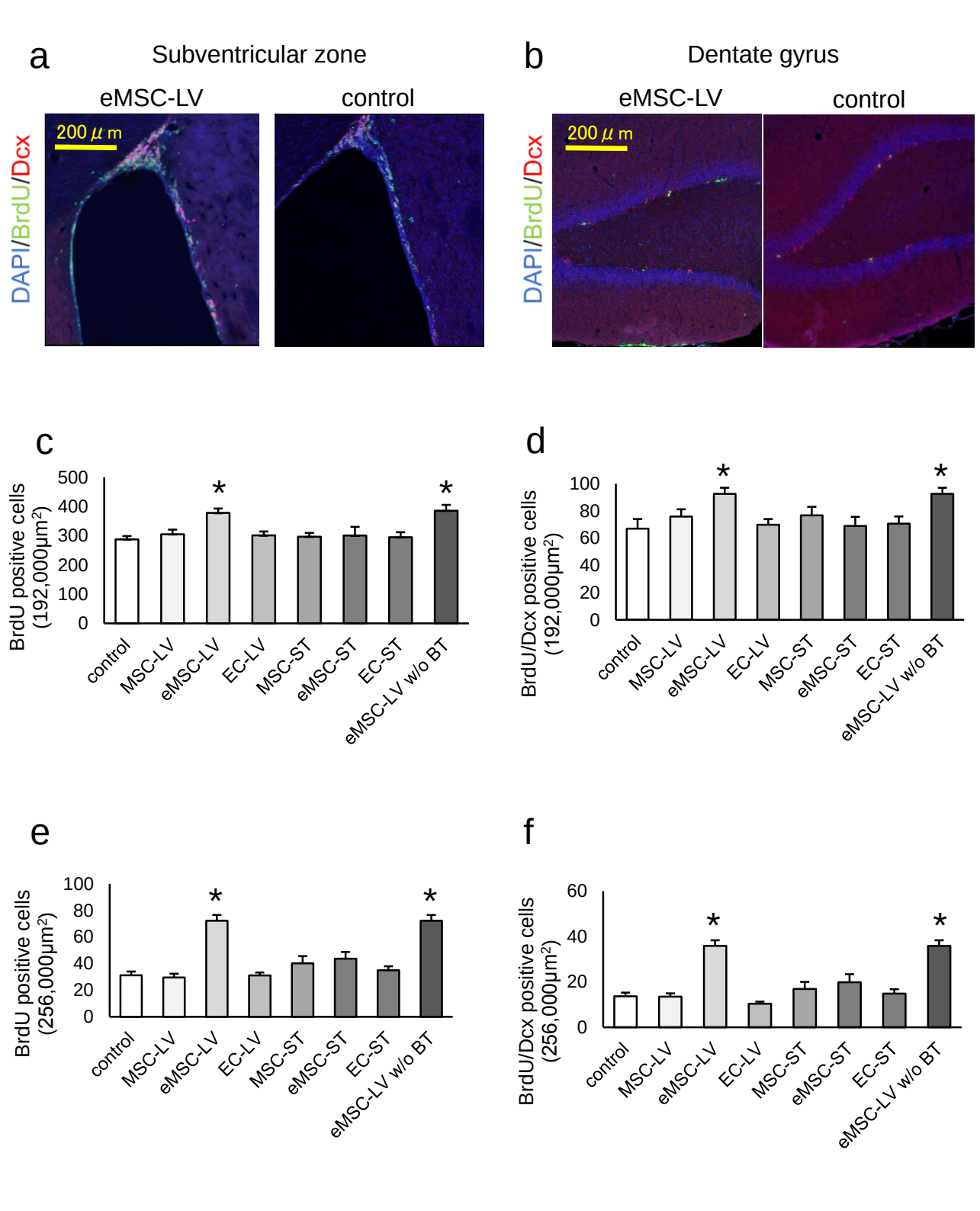
control
MSCs-LV
eMSCs-LV
EC-LV
MSCs-ST
eMSCs-ST
EC-ST
Fluoxetine
Wistar


a
Subventricular zone
eMSC-LV
control
200 μ m
DAPI/BrdU/Dcx
b
Dentate gyrus
eMSC-LV
control
200 μ m
DAPI/BrdU/Dcx
c
BrdU positive cells (192,000μm²)
500
400
300
200
100
0
*
*
control
MSC-LV
eMSC-LV
EC-LV
MSC-ST
eMSC-ST
EC-ST
eMSC-LV w/o BT
d
BrdU/Dcx positive cells (192,000μm²)
100
80
60
40
20
0
*
*
control
MSC-LV
eMSC-LV
EC-LV
MSC-ST
eMSC-ST
EC-ST
eMSC-LV w/o BT
e
BrdU positive cells (256,000μm²)
100
80
60
40
20
0
*
*
control
MSC-LV
eMSC-LV
EC-LV
MSC-ST
eMSC-ST
EC-ST
eMSC-LV w/o BT
f
BrdU/Dcx positive cells (256,000μm²)
60
40
20
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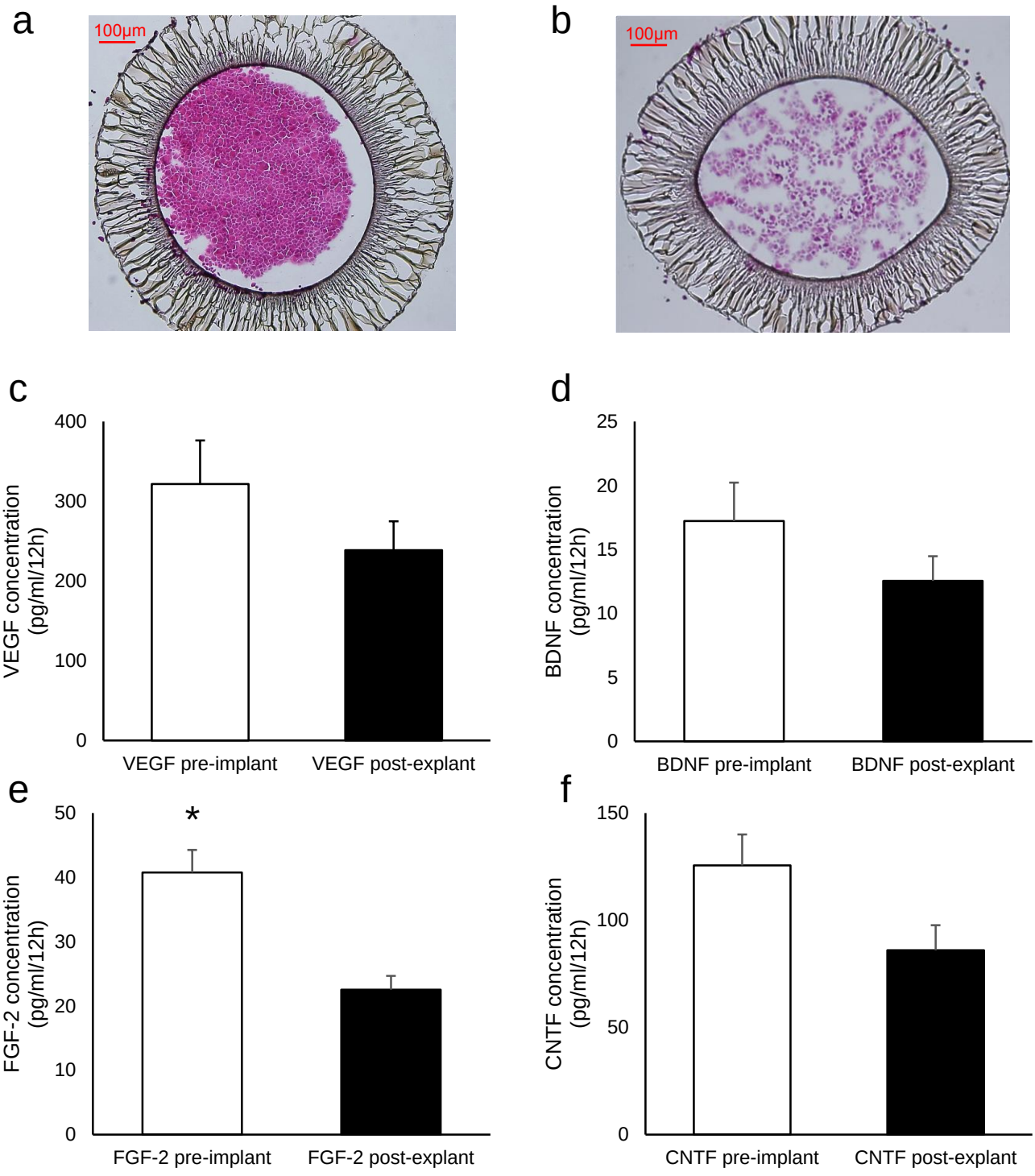
control
MSC-LV
eMSC-LV
EC-LV
MSC-ST
eMSC-ST
EC-ST
eMSC-LV w/o BT


a
100μm
b
100μm
c
VEGF concentration (pg/ml/12h)
400
300
200
100
0
VEGF pre-implant
VEGF post-explant
d
BDNF concentration (pg/ml/12h)
25
20
15
10
5
0
BDNF pre-implant
BDNF post-explant
e
FGF-2 concentration (pg/ml/12h)
50
40
30
20
10
0
*
FGF-2 pre-implant
FGF-2 post-explant
f
CNTF concentration (pg/ml/12h)
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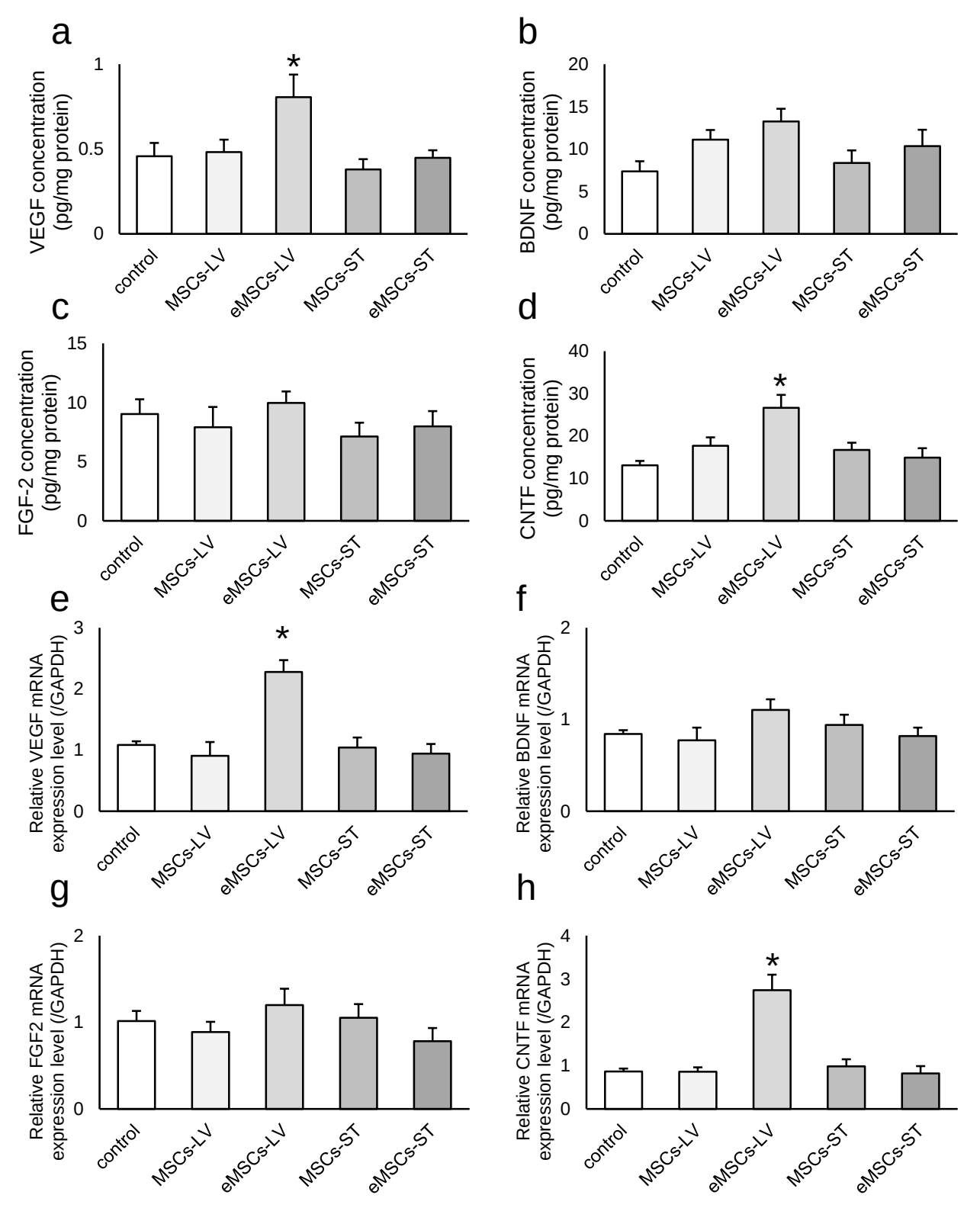
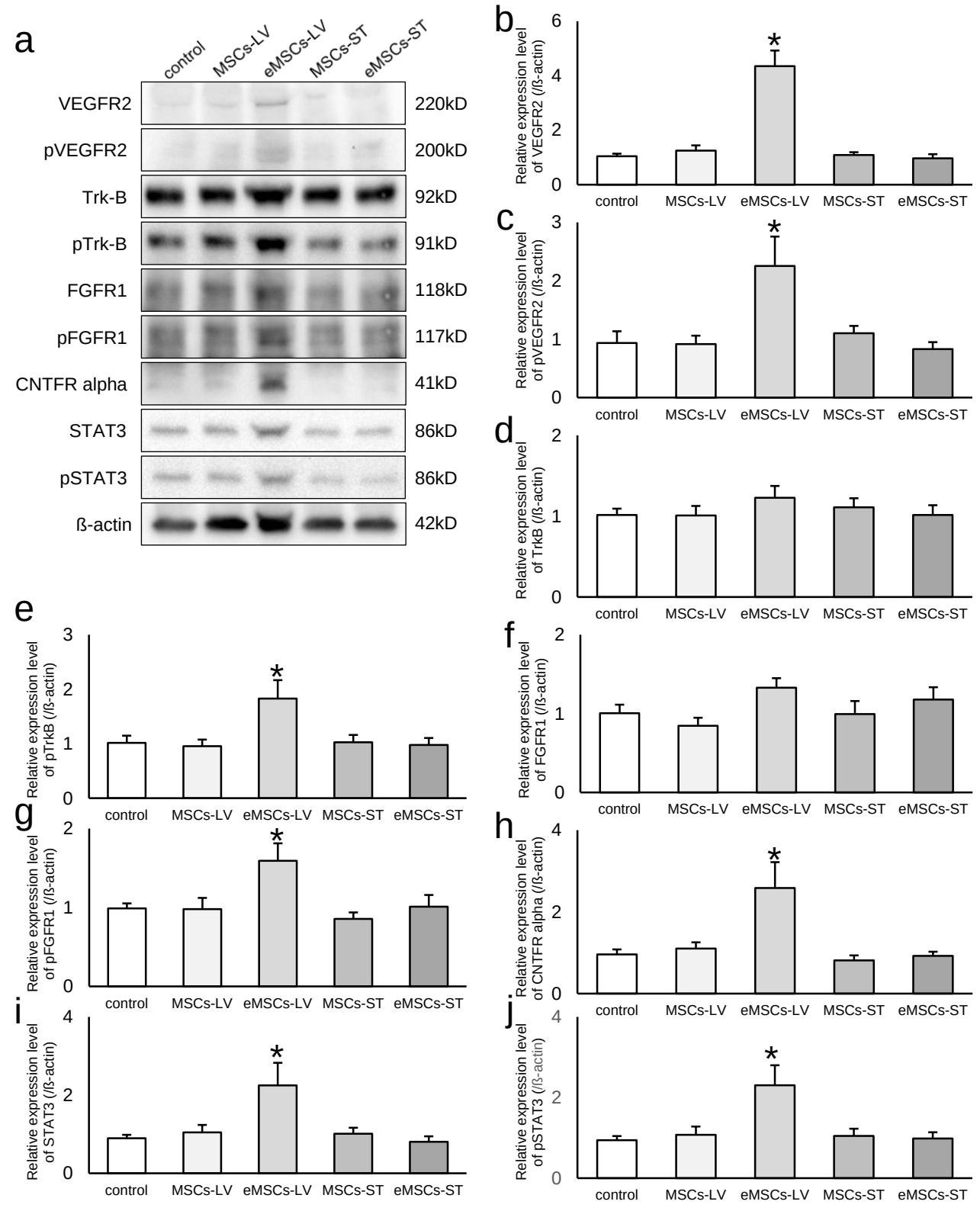
100
50
0
CNTF pre-implant
CNTF post-explant


a
VEGF concentration (pg/mg protein)
1
0.5
0
*
control
MSCs-LV
eMSCs-LV
MSCs-ST
eMSCs-ST
b
BDNF concentration (pg/mg protein)
20
15
10
5
0
control
MSCs-LV
eMSCs-LV
MSCs-ST
eMSCs-ST
c
FGF-2 concentration (pg/mg protein)
15
10
5
0
control
MSCs-LV
eMSCs-LV
MSCs-ST
eMSCs-ST
d
CNTF concentration (pg/mg protein)
40
30
20
10
0
*
control
MSCs-LV
eMSCs-LV
MSCs-ST
eMSCs-ST
e
Relative VEGF mRNA expression level (/GAPDH)
3
2
1
0
*
control
MSCs-LV
eMSCs-LV
MSCs-ST
eMSCs-ST
f
Relative BDNF mRNA expression level (/GAPDH)
2
1
0
control
MSCs-LV
eMSCs-LV
MSCs-ST
eMSCs-ST
g
Relative FGF2 mRNA expression level (/GAPDH)
2
1
0
control
MSCs-LV
eMSCs-LV
MSCs-ST
eMSCs-ST
h
Relative CNTF mRNA expression level (/GAPDH)
4
3
2
1
0
*
control
MSCs-LV
eMSCs-LV
MSCs-ST
eMSCs-ST


a
control
MSCs-LV
eMSCs-LV
MSCs-ST
eMSCs-ST
VEGFR2
220kD
pVEGFR2
200kD
Trk-B
92kD
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91kD
FGFR1
118kD
pFGFR1
117kD
CNTFR alpha
41kD
STAT3
86kD
pSTAT3
86kD
ß-actin
42kD
b
Relative expression level of VEGFR2 (/ß-actin)
c
Relative expression level of pVEGFR2 (/ß-actin)
d
Relative expression level of TrkB (/ß-actin)
e
Relative expression level of pTrkB (/ß-actin)
f
Relative expression level of FGFR1 (/ß-actin)
g
Relative expression level of pFGFR1 (/ß-actin)
h
Relative expression level of CNTFR alpha (/ß-actin)
i
Relative expression level of STAT3 (/ß-actin)
j
Relative expression level of pSTAT3 (/ß-actin)
control
MSCs-LV
eMSCs-LV
MSCs-ST
eMSCs-ST