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TITLE:

Culture of Neurospheres Derived from the Neurogenic Niches in Adult Prairie Voles

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KEYWORDS

prairie vole, cell culture, neurospheres, ventricular zone, dentate gyrus, neural stem cells

SUMMARY:

We established the conditions to culture neural progenitor cells from the subventricular zone and dentate gyrus of the adult brain of prairie voles, as a complementary in vitro study, to analyze the sex-dependent differences between neurogenic niches that could be part of functional plastic changes associated with social behaviors.

ABSTRACT:

Neurospheres are primary cell aggregates that comprise neural stem cells and progenitor cells. These 3D structures are an excellent tool to determine the differentiation and proliferation potential of neural stem cells, as well as to generate cell lines than can be assayed over time. Also, neurospheres can create a niche (in vitro) that allows the modeling of the dynamic changing environment, such as varying growth factors, hormones, neurotransmitters, among others. *Microtus ochrogaster* (prairie vole) is a unique model for understanding the neurobiological basis of socio-sexual behaviors and social cognition. However, the cellular mechanisms involved in

these behaviors are not well known. The protocol aims to obtain neural progenitor cells from the neurogenic niches of the adult prairie vole, which are cultured under non-adherent conditions, to generate neurospheres. The size and number of neurospheres depend on the region (subventricular zone or dentate gyrus) and sex of the prairie vole. This method is a remarkable tool to study sex-dependent differences in neurogenic niches in vitro and the neuroplasticity changes associated with social behaviors such as pair bonding and biparental care. Also, cognitive conditions that entail deficits in social interactions (autism spectrum disorders and schizophrenia) could be examined.

INTRODUCTION:

The prairie vole (*Microtus ochrogaster*), a member of the Cricetidae family, is a small mammal whose life strategy develops as a socially monogamous and highly sociable species. Both males and females establish an enduring pair bond after mating or long periods of cohabitation characterized by sharing the nest, defending their territory, and displaying biparental care for their progeny[1-4]. Thus, the prairie vole is a valuable model for understanding the neurobiological basis of socio-sexual behavior and impairments in social cognition[5].

Adult neurogenesis is one of the most paramount processes of neural plasticity that leads to behavioral changes. For example, our research group reported in male voles that social cohabitation with mating increased cell proliferation in the subventricular zone (VZ) and subgranular zone in the dentate gyrus (DG) of the hippocampus, suggesting that adult neurogenesis can play a role in the formation of pair bonding induced by mating in prairie voles (unpublished data). On the other hand, although the brain regions where new neurons are generated and integrated are well known, the molecular and cellular mechanisms involved in these processes remain undetermined due to technical drawbacks in the whole brain model[6]. For instance, the signaling pathways controlling gene expression and other cellular activities have a relatively short activation period (detection of phosphoproteome)[7]. One alternative model is isolated and cultured adult neural stem cells or progenitor cells to elucidate molecular components involved in adult neurogenesis.

The first approach to maintain in vitro neural precursors from adult mammal (mouse) brain was the assay of neurospheres, which are cellular aggregates growing under non-adherent conditions which preserve their multipotent potential to generate neurons, as well as astrocytes[8-10]. During their development, there is a selection process where only the precursors will respond to mitogens such as the Epidermal Growth Factor (EGF) and Fibroblast Growth Factor 2 (FGF2) to proliferate and generate neurospheres[8-10].

To our knowledge, no protocol is reported in the literature to obtain adult neural progenitors from prairie voles. Here, we established the culture conditions to isolate neuronal progenitors from neurogenic niches and their in vitro maintenance through the neurosphere formation assay. Thus, experiments can be designed to identify the molecular and cellular mechanisms involved in proliferation, migration, differentiation and survival of the neural stem cells and progenitors, processes that are still unknown in the prairie vole. Moreover, elucidating in vitro differences in the properties of the cells derived from the VZ and DG could provide information about the role

of neurogenic niches in neural plasticity associated with changes in socio-sexual behavior and cognitive behaviors, and deficits in social interactions (autism spectrum disorder and schizophrenia), which could also be sex-dependent.

PROTOCOL:

The study was approved by the Research Ethics Committee of the Instituto de Neurobiología, Universidad Nacional Autónoma de México, Mexico and Instituto Nacional de Perinatologia (2018-1-163). The reproduction, care and humane endpoints of the animals were established following the Official Mexican Standard (NOM-062-Z00-1999) based on the "Ley General de Salud en Materia de Investigación para la Salud" (General Health Law for Health Research) of the Mexican Secretaria of Health.

1. Solutions and stocks preparation

1.1. Prepare an N2 culture medium with 485 mL of Dulbecco's Modified Eagle Medium-F12 (DMEM-F12), 5 mL of N2 supplement (100x), 5 mL of glutamine supplement (100x) and 5 mL of antibiotic-antimycotic (100x).

1.2. Prepare a B27 culture medium with 480 mL of Neurobasal medium, 10 mL of B27 supplement (50x), 5 mL of glutamine supplement, and 5 mL of antibiotic-antimycotic (100x).

1.3. Reconstitute collagenase powder in 1x PBS (Phosphate-buffered saline) to obtain aliquots with an activity of 100 units/µL (1000x) and store at -20 °C. Notice, collagenase activity depends on the lot number of the companies.

1.4. Prepare dispase stock aliquots by dissolving 5 mg of dispase powder in 1x PBS (50 mg/mL). Store at -20 °C.

1.5. Prepare an enzymatic solution with 100 mL of DMEM-F12 medium, 50 µL of stock collagenase (100 units/µL) to have a final concentration of 50 U/mL and 333 µL of stock dispase (50 mg/mL) to have a final concentration of 0.33 mg/mL.

1.6. To prepare a washing solution, to 1,000 mL of 1x PBS, add 0.4766 g of HEPES (final concentration 2 mM), 3.6 g of D-glucose (final concentration 20 mM) and 2.1 g of $NaHCO_3$ (final concentration 25 mM).

1.7. Prepare poly-L-ornithine stock aliquots (1 mg/mL) using sterile water and store at -20 °C.

1.8. Prepare a working solution of poly-L-ornithine. Dilute a stock aliquot (1mg/mL) in 49 mL of sterile water for a final concentration of 20 µg/mL.

1.9. Prepare a working solution of laminin. Dilute 25 µL of laminin (1 mg/mL original stock) in 5 mL of sterile water for a final concentration of 5 µg/mL.

NOTE: After preparation, filter the culture media, working and stock solutions to avoid contamination. Use a syringe or bottle-top vacuum filters (polyethersulfone membrane with a 0.2 µm pore size). The culture media and work solutions can be stored for up to 30 days at 4 °C, while the stocks can be stored for up to four months at -20 °C.

2. Preparation before starting the microdissection

2.1 Sterilize surgical instruments by autoclaving or with a hot glass bead dry sterilizer.

2.2 Clean the microdissection surface area under strict aseptic and antiseptic conditions (e.g., with ozonized water).

NOTE: The timing of microdissection of both neurogenic niches from each vole brain is approximately 30 min. Working with 1-4 animals for the entire procedure is recommended.

3. Extraction of the whole brain

3.1 Anesthetize the adult vole (12-16 weeks) with an overdose of pentobarbital (6.3 mg/animal) through intraperitoneal injection. Verify the depth of anesthesia by the absence of pedal reflex in response to a firm toe pinch.

3.2 Once the vole is entirely anesthetized, induce euthanasia by decapitation and recover the head.

3.3 Dissect the skin from the skull with scissors, making a caudal-rostral incision (15 mm long) to expose the skull.

3.4 Cut the occipital and interparietal bones and trace an incision into the skull along the sagittal and parietal sutures.

3.5 Make a hole in the skull at the junction of frontal and parietal bones using scissors, being very careful not to damage the brain tissue.

3.6 To expose the brain, remove the remaining cranium fragments that cover both brain hemispheres with sharp-pointed tweezers.

3.7 Use a stainless-steel spatula to lift the entire brain from the cranial base.

3.8 Collect the brain into a centrifuge tube (50 mL) with 20 mL of cold wash solution.

3.9 Wash the brain twice with the cold wash solution.

4. Microdissection of the neural tissue

4.1 Place a Petri dish on a surface surrounded by ice.

4.2 Deposit the brain on the dish and add 20 mL of cold wash solution.

4.3 With a scalpel, in the coronal plane, divide the brain into two blocks of tissue (rostral and caudal). As a neuroanatomical reference, perform the coronal cut at Bregma level in the anterior-posterior axis[11] (**Figure 1A**, solid line).

4.4 From the rostral block, extract the VZ tissue (**Figure 1B**), while from the caudal block remove the DG (**Figure 1C**).

4.5 Dissect the VZ under a stereo microscope.

4.5.1 With a Dumont forceps, hold one of the hemispheres; then, insert, at the height of the ventricle, the fine tips of a second Dumont forceps under the tissue that lines the caudate-putamen (**Figure 2A**).

4.5.2 Open the forceps along the dorsoventral axis to separate the tissue.

4.5.3 Collect the VZ tissue per individual in a centrifuge tube with 2 mL of cold wash solution. Do not pool the tissue of more than two animals.

4.5.4 Repeat the microdissection in the other hemisphere.

4.5.5 Store the tube containing the bilateral VZ tissue on ice and continue dissecting the DG.

4.6 Dissect the DG from the caudal block under a stereo microscope.

4.6.1 With a scalpel, make a coronal cut into the block to obtain two slices, in which the hippocampal formation is observed. As a landmark, the cut is made at -2 mm Bregma coordinates in the anterior-posterior axis according to the mouse brain atlas[11] (**Figure 1A**, dotted line and **Figure 1C**).

4.6.2 With a Dumont forceps, hold one of the slices, and with fine-point Dumont forceps make a horizontal cut between DG and CA1 and then perform a vertical incision between the DG and CA3 to separate the DG (**Figure 2B**).

4.6.3 Repeat the dissection in the first slice of the other hemisphere.

4.6.4 Repeat the dissection in both hemispheres in the second slice.

4.6.5 Collect the four DG pieces of each vole in a centrifuge tube. Do not pool the DG tissue of more than two animals.

NOTE: If dissection of more than one animal is required, store the centrifuge tubes with the VZ or DG tissue on ice while continuing to dissect the rest of the brains. Remove all blood vessels that cover the brain tissue while dissecting. If the vessels are not discarded, the culture could be mixed with an excess of erythrocytes and disturb neurosphere formation.

5. Isolation of neural cells

5.1 Place the centrifuge tubes inside the biosafety cabinet and wait about 10 min for the tissue fragments to precipitate by gravity.

5.2 Remove the wash solution and add 1 mL of the warm enzymatic solution to each tube.

5.3 Incubate the tubes at 37 °C for 10 min.

5.4 Disintegrate the tissue fragments; pipette up and down with a 1 mL tip. Do not pipette more than 30x.

5.5 Carry out a second incubation of 10 min at 37 °C.

5.6 At the end of the second incubation, pipette to break up the tissues. Do not pipette more than 30x.

NOTE: After pipetting, the tissue fragments should be completely disintegrated; if they are not disintegrated, incubate for another 10 min at 37 °C and re-pipette. The digestion period should not exceed 30 min.

5.7 Add 9 mL of N2 medium per tube to dilute the enzymatic treatment.

5.8 Centrifuge the tubes at 200 *x g* for 4 min at room temperature.

5.9 Discard the supernatant and wash with 10 mL of N2 medium.

5.10 Centrifuge under the same conditions as step 5.8.

5.11 Remove the supernatant from each tube and resuspend the cell pellets of the VZ and DG in 2 mL and 1 mL of the B27 medium, respectively.

5.12 To remove any non-disintegrated tissue, filter each cellular suspension using a cell strainer (size 40 µm).

6. Neurospheres formation

6.1 Culture the cells passed through the strainer into an ultra-low attachment, 24-well plate. Use two wells for the VZ and one well for the DG (1 mL of B27 medium/well).

6.2 Add 20 ng/mL of FGF2 and 20 ng/mL of EGF to each well (final concentration 1x).

6.3 Incubate at 37 °C, 5% CO_2 and high humidity (90-95%). Do not disturb for 48 h (day 1 and day 2 of culture, D1-D2).

6.4 On the third day (D3), remove half of the culture medium and replace it with fresh B27 medium (500 µL per well) supplemented with double concentration (2x) of growth factors.

6.5 Repeat every third day, change the culture medium (half of it) and replace it with a fresh B27 medium supplemented with double concentration (2x) of growth factors.

6.6 On days when it is not necessary to change the culture medium, add growth factors to a final concentration of 1x.

6.7 Ensure that the neurospheres are formed around D8-D10.

6.8 At the D10, change the complete culture medium to remove all debris.

6.8.1 Collect the medium and neurospheres individually of each well in centrifuge tubes.

6.8.2 Incubate for 10 min at room temperature. This procedure allows neurospheres precipitation by gravity.

6.8.3 Remove the supernatant and resuspend in 1 mL of fresh B27 medium supplemented with growth factors.

6.8.4 Place the neurospheres back into the same ultra-low attachment plate and incubate at 37 °C, 5% CO_2.

6.9 From D10 to D15, continue changing half of the medium and adding growth factors.

7. Passage of the neurospheres

7.1 At D15 of the primary culture, collect the neurospheres into centrifuge tubes using 1 mL pipette. Cut the pipette tip to increase the size of the opening to avoid damage to the neurospheres.

7.2 Incubate for 10 min at room temperature. Neurospheres precipitate by gravity.

7.3 Remove the medium and add 1 mL of the cell detachment medium per tube.

7.4 Incubate the tubes for 7 min at 37 °C.

7.5 Pipette up and down with a 1 mL tip to dismantle the neurospheres.

7.6 Dilute the cell detachment medium with 3 mL of B27 medium per tube.

7.7 Centrifuge the cell suspension for 5 min at 200 *x g*.

7.8 Discard the supernatant and resuspend each cell pellet with a fresh B27 medium supplemented with growth factors.

7.8.1 Resuspend the VZ-derived cells in 4 mL of medium and the DG-derived cells in 2 mL of medium.

7.9 Culture the cells (passage 1) in a new ultra-low attachment plate by doubling the number of wells that were used in the primary culture (4 and 2 wells for VZ and DG, respectively).

7.10 Change half of the medium every third day and add growth factors daily.

7.11 After 10 days (D10) in passage 1, change to adherent conditions in the next passage.

8. The passage in adherent conditions

8.1 Before carrying out the passage 2, prepare coated plates with poly-L-ornithine and laminin.

8.1.1 In 24-well plates, add 500 µL of 1x poly-L-ornithine (20 µg/mL) per well. Incubate at 37 °C overnight.

8.1.2 Remove the poly-L-ornithine and wash 4x with 1x PBS (500 µL/well).

8.1.3 Add 200 µL (minimum volume to cover the surface of a single well) of 1x laminin (5 µg/mL) per well and incubate for 2-3 h at 37 °C before cultivating the cells.

8.2 Collect the neurospheres with 1 mL pipette with cut tips into a centrifuge tube.

8.3 Incubate for 10 min at room temperature to precipitate the neurospheres by gravity.

8.4 Discard the supernatant and resuspend the neurospheres in fresh B27 medium without growth factors.

8.5 Aspirate the laminin from the coated plate and deposit the neurospheres into the wells using 1 mL pipettes with cut tips.

NOTE: Prevent coated wells from drying out between laminin removal and plating neurospheres.

8.6 Divide the culture into two conditions:

8.6.1 Maintain differentiated neurospheres for 6 days (D6). Change the medium every third day and add growth factors daily.

8.6.2 Observe differentiation of the neurosphere-derived cells by 12 days (D12). Change the medium every third day without growth factors.

NOTE: At the end of D6 for undifferentiated or D12 for differentiation conditions, the cells can be used for conventional immunohistochemistry, cell sorting analysis, 5-Ethynyl-2´-deoxyuridine (EdU) staining, RNA extraction, among others.

REPRESENTATIVE RESULTS:

Neurospheres were formed from neural stem cells isolated from the VZ and DG of both female and male adult prairie voles. About 8-10 days after starting the culture, cells should have formed the neurospheres. Note that the plate may contain debris in the primary culture (**Figure 3A**). However, in passage 1 the culture should only consist of neurospheres (**Figure 3B**).

A higher number of neurospheres were obtained from the female VZ as compared with the male VZ and DG of both females and males (**Figure 4A**). These data suggest that the number of neurospheres obtained depends on the proliferative zone and the vole sex. Once the neurospheres appeared (D8-D10), they were maintained for another seven days in culture, and their growth was monitored during this period. The diameter of the neurospheres was measured on D8, D11, and D14 (**Table 1** and **Figure 4B**). The neurosphere's size (diameter) increased progressively according to the days of culture for male and female voles in both neuronal regions. Neurospheres derived from the male brains were smaller in comparison to the neurospheres derived from the female brain in both neurogenic areas (**Figure 4B**).

After 15 days of primary culture on floating conditions, the neurospheres were expanded in passage 1 under the same conditions. For the subsequent passage 2, the cells grew in adhesive culture, although they were able to adhere since passage 1. Adhered neurospheres were characterized at day six (D6) in the presence of growth factors (undifferentiated condition, **Figure 5A**) or instead until day 15 (D15) without growth factors (differentiated condition, **Figure 5B**).

At D6 under undifferentiation conditions, the neurosphere-derived cells expressed nestin (a marker for neural progenitors) (**Figure 6**). Also, it was possible to identify doublecortin (DCX) positive cells (migration cells) and the proliferation marker Ki67, which indicate the presence of either neuronal precursors or immature neurons. However, the lack of colocalization of Ki67 with DCX suggests the presence of postmitotic neuroblasts (**Figure 7**). Finally, at D15 under differentiation conditions, mature neurons (MAP2-positive cells) were found, as well as cells with the glial phenotype (GFAP-positive cells), which demonstrates differentiation potential of the isolated cells (**Figure 8**).

FIGURE AND TABLE LEGENDS:

Figure 1: Dorsal view of an adult vole brain and its neurogenic regions. (**A**) The solid line at Bregma level was the anatomical reference to separate the brain into two blocks, rostral and caudal. The dotted line was the reference to divide the caudal block to obtain two slices containing the DG. (**B**) Coronal view of the neuronal regions exposed with the first incision, where the VZ is located. (**C**) Coronal view of the anatomical regions exposed with the second incision, where the DG is located.

Figure 2: Anatomical references for the dissection of neurogenic regions. (**A**) Scheme and photograph of the coronal section from the rostral block showing the VZ location (dotted line). (**B**) Scheme and photograph of the coronal section from the caudal block showing the DG dissection. CPu, caudate putamen; V, ventricle; VZ, ventricular zone, DG, dentate gyrus; CA1 and CA3, regions of the hippocampus.

Figure 3: Representative micrographs of neurospheres culture derived from neurogenic niches of the adult prairie vole. (**A**) Primary culture of neurospheres isolated from the VZ of female voles at D10. (**B**) Passage 1 of neurospheres derived from the VZ of female voles at D10. Scale bars = 200 µm. n= 3 for each neurogenic region and sex of the vole.

Figure 4: The number and size of neurospheres depended on both sex and neurogenic source. (**A**) The number of neurospheres in primary culture obtained from VZ and DG in both female and male voles at D10. Data were analyzed with a one-way ANOVA followed by a Tukey's post hoc tests. Significant differences were found between the female VZ and the rest of the groups, ***$p<0.001$. (**B**) The diameter of the neurospheres throughout D8-D14 in the primary culture depended on the vole sex. Data were analyzed with a two-way ANOVA followed by Tukey's post hoc tests. Intra-group comparisons (differences within the same group) showed an increase in the neurosphere size between D8 vs. D11 and D14, (*$p<0.05$, ***$p<0.001$, ****$p<0.0001$); and D11 vs. D14 (+++ $p<0.001$) in the VZ and DG of female and male voles. Inter-group comparison (differences between groups in the same region) showed that female VZ and DG neurospheres are larger than male neurospheres at D11 and D14. ### $p<0.0001$. VZ was obtained from males and female voles (n=3, per group). 15 female and 10 male neurospheres were analyzed. DG was obtained from males and female voles (n=3, per group). 8 female neurospheres and 5 male neurospheres were processed.

Figure 5: Representative images of neurospheres derived from the female VZ cultured in adhesion conditions in passage 2. (**A**) Neurospheres adhered in passage 2 with growth factors at D2. (**B**) Neurosphere-derived cells adhered in passage 2 without growth factors at D10. Scale bar = 200 µm.

Figure 6: Expression of nestin in neurospheres. Representative, epifluorescence-microscopy images of nestin-positive cells derived from the VZ of both female and male adult brains at the undifferentiated stage. Scale bars = 50 µm.

Figure 7: Expression of DCX and Ki67 in neurospheres. Representative epifluorescence-microscopy images of DCX-, Ki67-positive cells and merge derived from the VZ of both adult female and male brains at the undifferentiated stage. Scale bars = 25 µm.

Figure 8: Expression of MAP2 and GFAP in neurospheres. Representative, epifluorescence-microscopy images of MAP2 (mature neurons) and GFAP (glial cells) positive cells derived from the VZ of both adult female and male brains at the differentiated stage. Scale bars = 50 µm.

Table 1: Quantification of the average size (diameter) of neurospheres isolated from neurogenic niches in the primary culture. Significant differences are shown in Figure 4. VZ was obtained from males and female voles (n=3, per group). Fifteen neurospheres from females and ten from males were analyzed. DG was obtained from males and female voles (n=3, per group). Eight female neurospheres and five male neurospheres were processed.

DISCUSSION:

A stage to obtain a neural stem cell culture is the digestion period with the enzymatic solution, which should not exceed more than 30 min because it might decrease cell viability. The neurospheres should emerge at 8-10 days after initial culture; if they do not emerge by day 12, discard the culture and repeat the experiment, reducing the digestion period. Another issue is the blood vessels that cover the brain tissue. They should be completely removed during the dissection because the excess of erythrocytes can interfere with the neurospheres formation.

This protocol allows expanding the floating neurospheres until passage 2 and changing to adherent conditions to evaluate the neurosphere-derived cells. However, it has limitations such as a decrease in the neurogenic potential, which switches to gliogenic differentiation at successive passages as an adaptation response to in vitro conditions[12]. For this reason, we recommended characterizing the neurospheres in the primary culture and passage 1, and continue with the next passage only if it is required to expand the cells for experiments that do not involve elucidating differences due to the origin.

Interestingly, intrinsic differences can be found in the primary culture of neurospheres as a result of the neuroanatomical (VZ or DG) or sex-dependent (females or males) source. Thus, the number and diameter of neurospheres derived from both neurogenic regions of females are higher in comparison with males. This could be a functional difference in the female brain niche compared to that in males, which molecular mechanisms can be studied in vitro with this assay. Cell culture of neurospheres derived from the adult brain vole is a valuable tool that could help resolve discrepancies between studies in vivo. For example, Fowler and coworkers reported that social isolation for 48 h induces an increase in 5-Bromo-2'-deoxyuridine (BrdU)-positive cells in the VZ, without affecting the DG[6]. In contrast, Lieberwirth et al.; demonstrated a decrease in cell proliferation in the DG[13]. Furthermore, in vitro culture can be a model for evaluating the molecular mechanisms in neurogenic regions that could be associated with behavioral changes in a social model such as the prairie vole. For example, it has been suggested that exposure to newborns induces, in both non-parental and parental voles, an increase of BrdU- positive cells in the DG[14]. The findings of this study can be confirmed using our cell culture protocol with BrdU

labeling. However, although most studies on voles and other mammals use BrdU labeling to identify new cells, a disadvantage is that the labbeling might change depending on the injected doses[15]. EdU, another thymidine analog, is an ideal alternative to identify cells under the cell cycle phase in vitro cultures. In the same experiment, it is possible to have several periods for the incorporation of EdU, and unlike BrdU, DNA denaturation or incubation with antibodies it is not necessary for its detection. Also, EdU-positive cells can be assessed for co-localization with markers to identify the cell-division cycle (Ki67) and determine their phenotype using markers of neural stem cells or progenitors (Nestin, Sox2 and Pax6).

The neurospheres culture can be established as a model to study the effect of hormones, small molecules or drugs in the proliferation rate, neurogenesis and epigenetic modifications in the neural stem cells and progenitors of prairie voles. For example, previous studies have suggested the role of the stress hormones (like corticosterone) and estrogens in the regulation of adult neurogenesis in prairie voles, but the underlying regulatory mechanisms are unknown[6].

Finally, autism spectrum disorders (ASD) and schizophrenia (SZ) are related to impairments in social cognition[16,17]. Interestingly, oxytocin and arginine-vasopressin have a fundamental role in social and emotional behavior, and gene expression variations in their receptors (OXTR and vasopressin 1a (V1AR), respectively) are associated with both ASD and SZ[18-21]. Moreover, alteration in neurogenesis and neural migration during neurodevelopment are implicated in the physiopathology of these behavioral disorders[22-24]. Thus, we propose to analyze the molecular mechanisms mediated by these hormones on neurogenesis, neural migration and other cellular events whose alterations are related to neurological disorders using prairie vole cell culture in vitro model due OXTR and V1AR receptors are found in the prairie vole hippocampus[25,26].

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DISCLOSURES:

The authors have nothing to disclose.

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580

Figure 1

Click here to access/download;Figure;Figure 1.psd

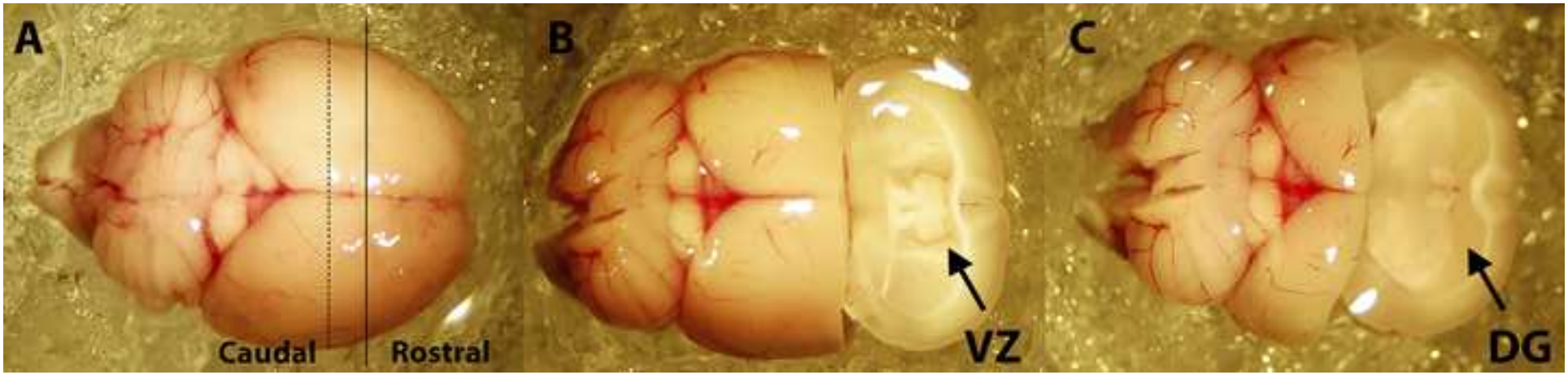

Figure 2

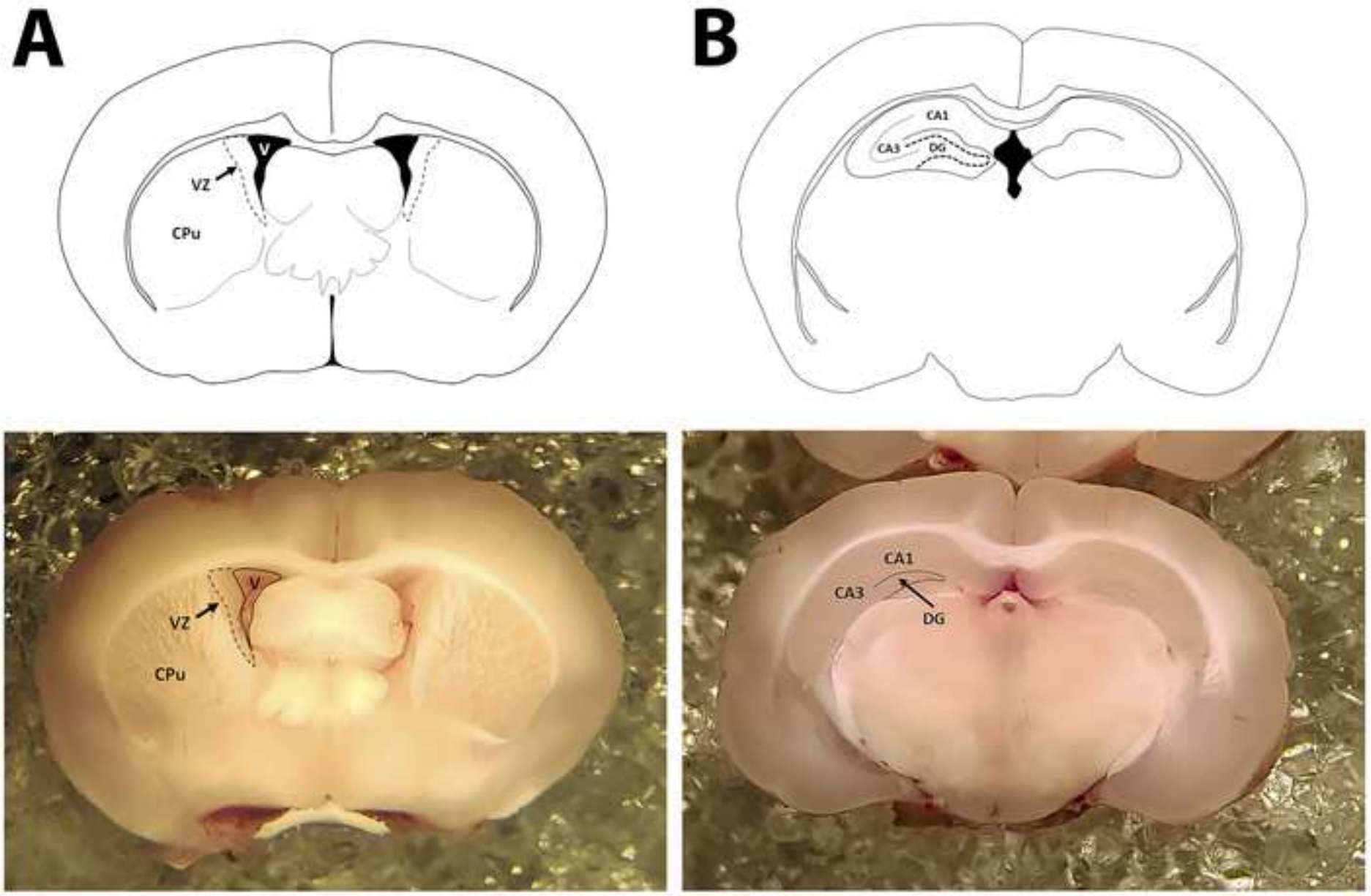

Figure 3

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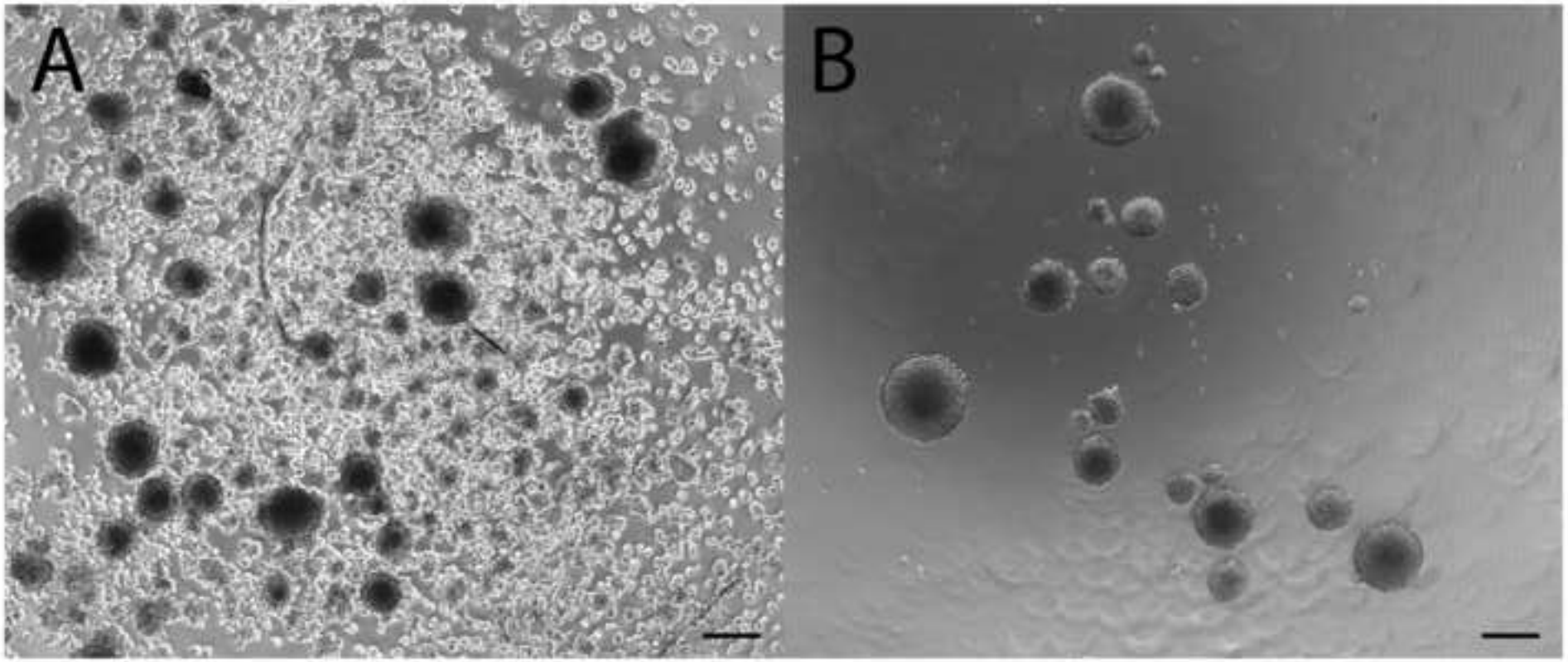

Figure 4

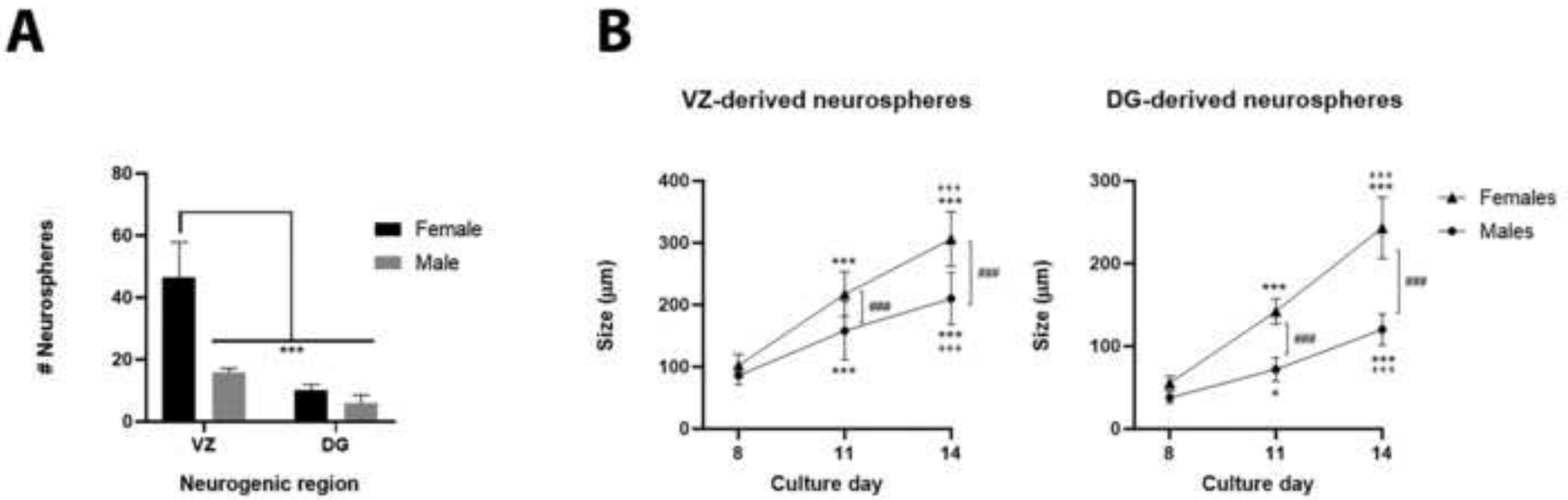

Figure 5

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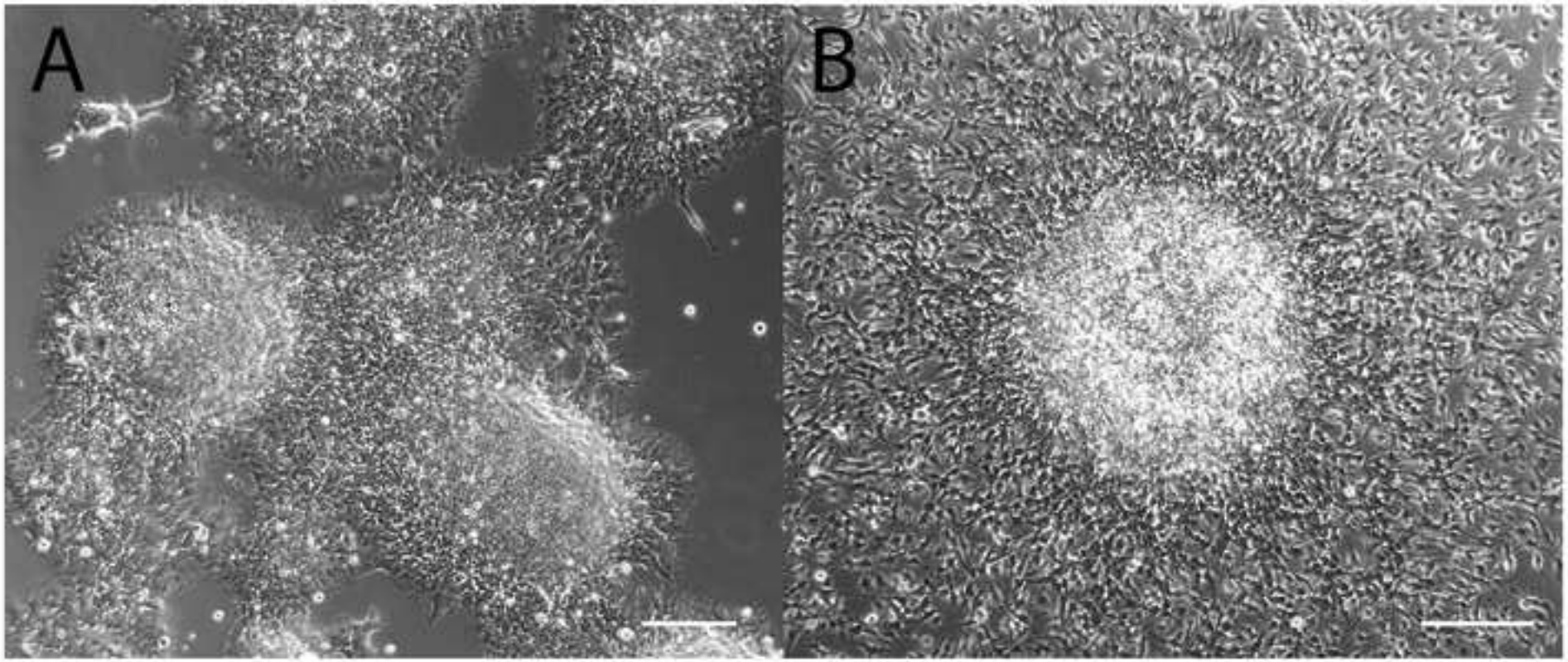

Figure 6

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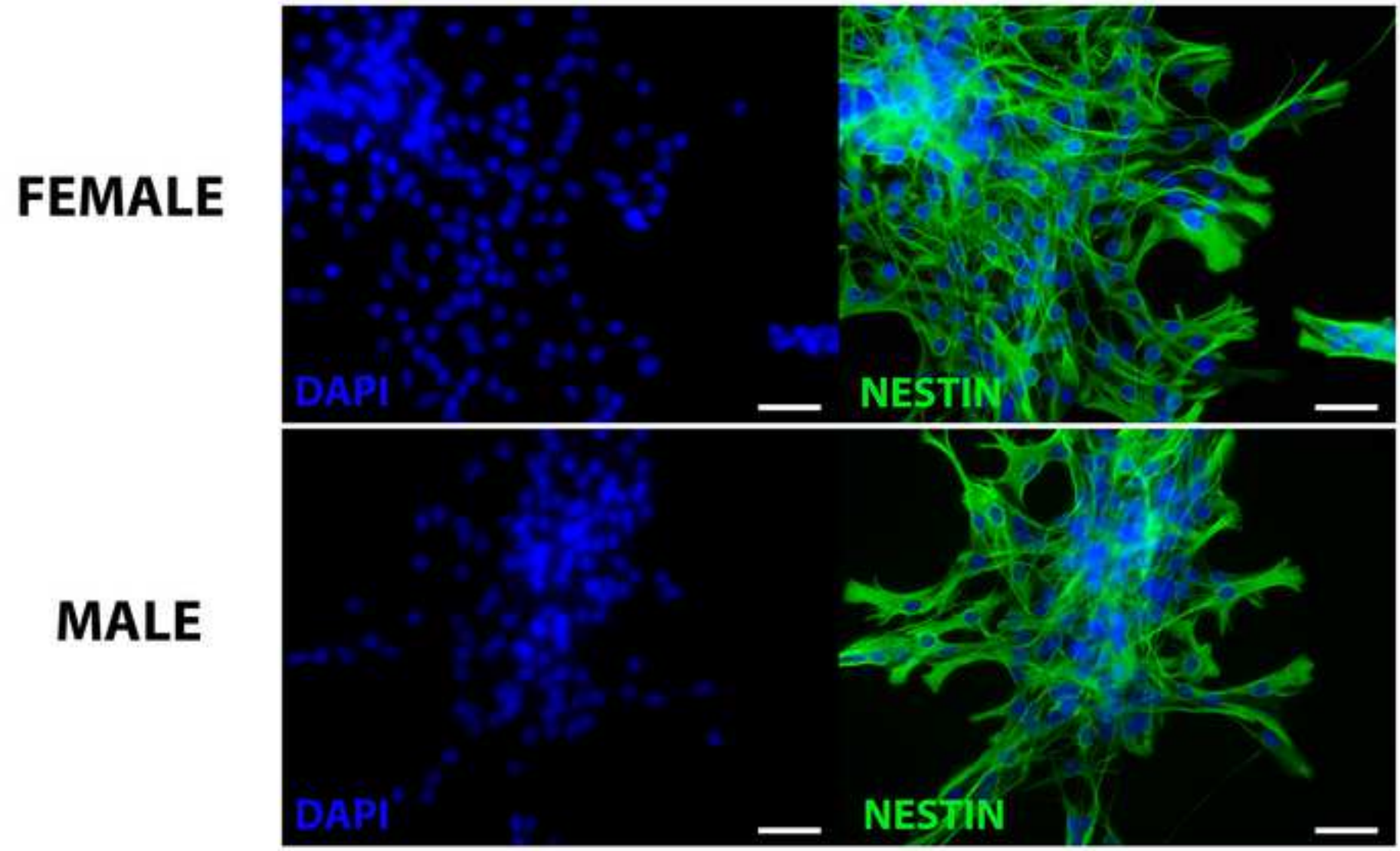

Figure 7

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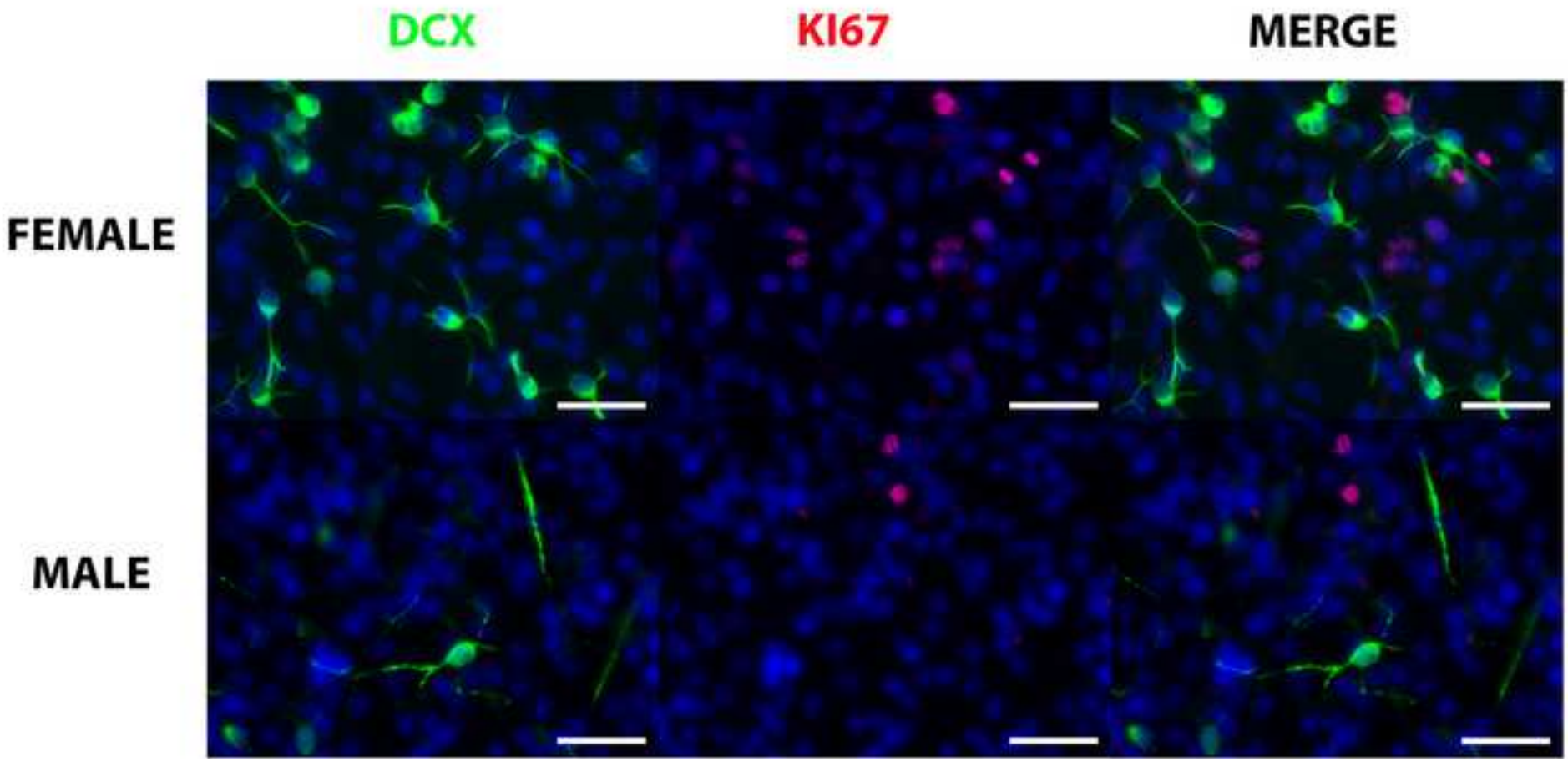

Figure 8

Click here to access/download;Figure;Figure 8.psd

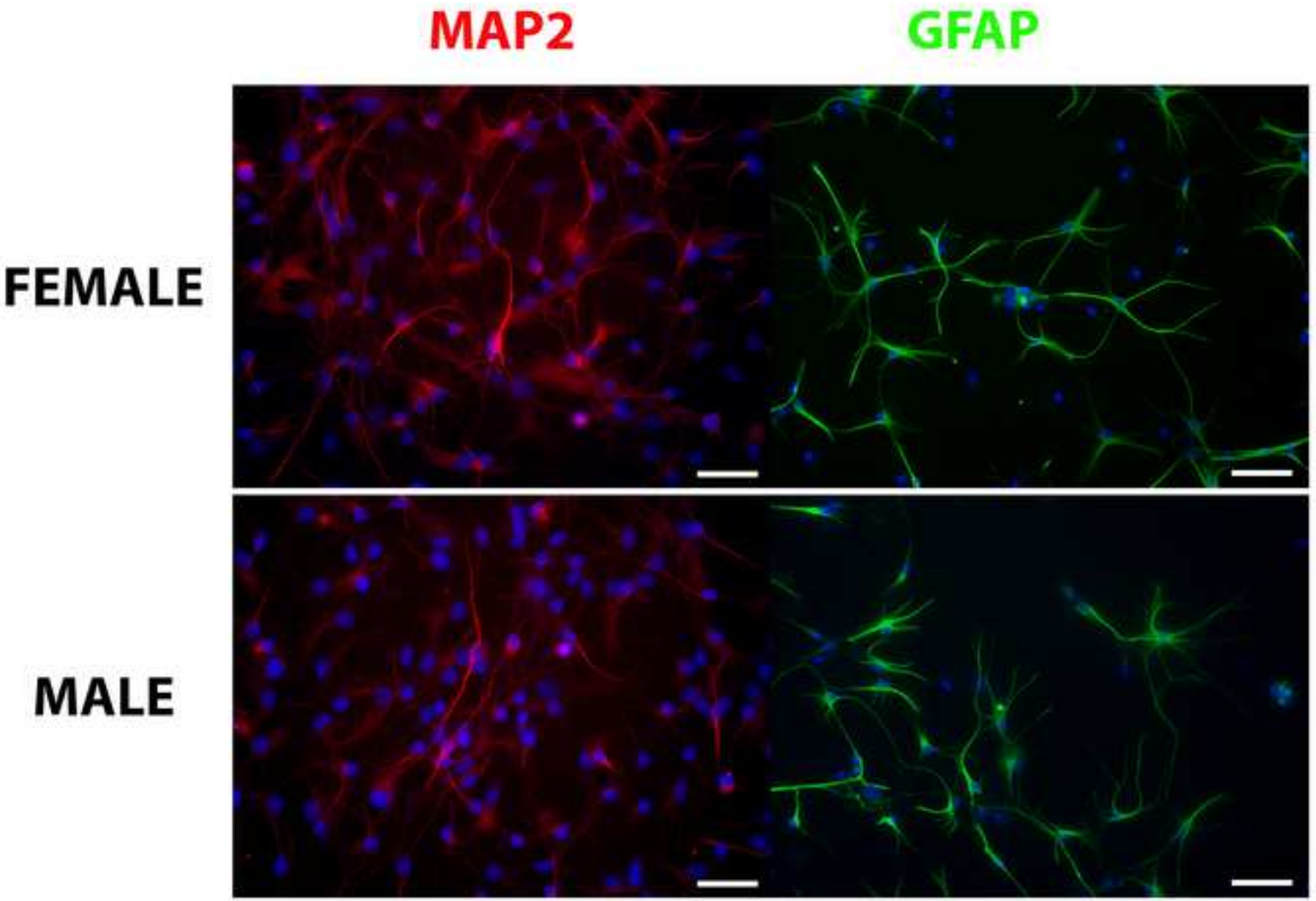

Table 1

Size of neurospheres (µm)					
VZ			DG		
Days of culture	Sex	Mean ± SD	Days of culture	Sex	Mean ± SD
D8	F	102.1±18.2	D8	F	55.3±8.5
	M	86.5±15.1		M	37.6±6.8
D11	F	217.3±35.7	D11	F	142.1±15.4
	M	158.9±47.2		M	71.8±14.4
D14	F	306.6±44.4	D14	F	243.8±37.4
	M	210.8±42.3		M	120.2±19.1

Table of Materials

Name of Material/ Equipment	Company
Antibodies	
Anti-Nestin	GeneTex
Anti-Doublecortin	MERCK
Anti-Ki67	Abcam
Anti-MAP2	GeneTex
Anti-GFAP	SIGMA
Goat Anti-Mouse Alexa Fluor 488	Thermo Fisher Scientific
Goat Anti-Rabbit Alexa Fluor 568	Thermo Fisher Scientific
Goat Anti-Guinea Pig Alexa Fluor 488	Thermo Fisher Scientific
Culture reagents	
Antibiotic-Antimycotic	Thermo Fisher Scientific/Gibco
B-27 supplement	Thermo Fisher Scientific/Gibco
Collagenase, Type IV	Thermo Fisher Scientific/Gibco
Dispase	Thermo Fisher Scientific/Gibco
DMEM/F12, HEPES	Thermo Fisher Scientific/Gibco
Glucose	any brand
GlutaMAX	Thermo Fisher Scientific/Gibco
HEPES	any brand
Mouse Laminin	Corning
N-2 supplement	Thermo Fisher Scientific/Gibco
$NAHCO_3$	any brand
Neurobasal	Thermo Fisher Scientific/Gibco
Phosphate-Buffered Saline (PBS)	Thermo Fisher Scientific/Gibco
Poly-L-ornithine hydrobromide	Sigma-Aldrich
Recombinant Human EGF	Peprotech
Recombinant Human FGF-basic	Peprotech
StemPro Accutase Cell Dissociation Reagent	Thermo Fisher Scientific/Gibco
Disposable material	
24-well Clear Flat Bottom Ultra-Low Attachment Multiple Well Plates	Corning/Costar

24-well Clear TC-treated Multiple Well Plates	Corning/Costar
40 µm Cell Strainer	Corning/Falcon
Bottle Top Vacuum Filter, 0.22 µm pore	Corning
Non-Pyrogenic Sterile Centrifuge Tube	any brand
Non-Pyrogenic sterile tips of 1,000 µl, 200 µl and 10 µl.	any brand
Sterile cotton gauzes	
Sterile microcentrifuge tubes of 1.5 mL	any brand
Sterile serological pipettes of 5, 10 and 25 mL	any brand
Sterile surgical gloves	any brand
Syringe Filters, 0.22 µm pore	Merk Millipore

Equipment and surgical instruments

Biological safety cabinet
Dissecting Scissors
Dumont Forceps
Motorized Pipet Filler/Dispenser
Micropipettes
Petri Dishes
Scalpel Blades
Stainless-steel Spatula

Catalog Number	Comments/Description
	Antibody ID
GTX30671	RRID:AB_625325
AB2253	RRID:AB_1586992
ab66155	RRID:AB_1140752
GTX50810	RRID:AB_11170769
G3893	RRID:AB_477010
A-11029	RRID:AB_2534088
A-11036	RRID:AB_10563566
A-11073	RRID:AB_2534117
15240062	100X
17504044	50X
17104019	Powder
17105041	Powder
11330032	
	Powder, Cell Culture Grade
35050061	100X
	Powder, Cell Culture Grade
354232	1 mg/mL
17502048	100X
	Powder, Suitable for Cell Culture
21103049	
10010023	1X
P3655	Powder
AF-100-15	
AF-100-18B	
A1110501	100 mL
3473	

3526	
352340	Blue
431118	PES membrane, 45 mm diameter neck with conical bottom
SLGPR33RB	Polyethersulfone (PES) membrane, 33 mm diameter

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April 9, 2020

Dr. Vineeta Bajaj,
Review Editor
JoVE

Dear Dr. Bajaj enclosed you will find the revised version of our manuscript "**Culture of neurospheres derived from the neurogenic niches in adult prairie voles**" JoVE60551.

The changes suggested by the reviewers are highlighted in the new version . We thank the reviewers for their constructive comments that helped us improve our manuscript.

A detailed list of chcnages is included.

We hope that the new version of our manuscript is suitable for publication in JoVE.

Sincerely,

Dr. Wendy Portillo

Editorial Comments

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.1

Response: As suggested, the new version of the paper was revised by an English native speaker with scientific background.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points.

Response: The manuscript was formatted as requested.

3. Please provide an email address for each author.

Response: In this new version of the manuscript, the email address for each author was added.

4. Please provide at least 6 keywords or phrases.

Response: In this new version of the manuscript, six keywords are provided.

5. Please ensure that the summary is describes the goal of the protocol in 10-50 words.

Response: The summary describes the goal of the protocol in 49 words.

6. Please describe all abbreviation during the first time use.

Response: In this new version of the manuscript, all abbreviations are described.

7. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.

Response: The Abstract contains 199 words and describes the goal of the protocol.

8. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).

Response: The protocol text was revised to avoid personal pronouns.

9. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

Response: As suggested, we made the changes requested in the text.

10. The Protocol should contain only action items that direct the reader to do something in complete sentences.

Response: As suggested, the changes requested were made.

11. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

Response: As suggested, the changes requested in the text of our manuscript were made.

12. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

Response: In this new version of our manuscript, the recommendation was followed.

13. 1: Please either use complete sentences to describe the making of the solutions or make a separate table in .xlsx format to describe the solution preparation. If making a table, please do not embed in the text but upload it separately as .xlsx file to your editorial manager account.

Response: In this new version of our manuscript, we follow the recommendation to use complete sentences for solutions/stock preparations. Also, the instructions to prepare stock and working solutions are described (lines 106-110 and 117-121).

14. 3.1: How do you check for the depth of anesthesia?

Response: We added in line 136 the criteria for the depth of the anesthesia. In addition, we included in the line 96 the guideline NOM-062-Z00-1999 of the General Health Law for Health Research of the Mexican Secretaria of Health, where the pedal reflex (firm toe pinch) is mentioned as one of the most common indicators to assess the effect of the depth of anesthesia.

15. 3.3: How big is the incision?

Response: The incision size was 15 mm long (line 140).

15. Only one note can follow one step. Also notes cannot be filmed. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step.

Response: In this new version of the manuscript, a few 'Notes' were converted to 'Steps.'

17. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Response: The essential video steps are highlight in yellow.

18. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Response: In our manuscript no previously, published figures are included.

19. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Response: in this new version of the manuscript, we separate in the discussion the critical steps (line 361), limitations of the method (369), the significance and future applications (line 378)

20. Please do not abbreviate the journal titles in the references section.

Response: As suggested the complete journal title is now provided, in the references section.

21. Please sort the materials table in alphabetical order.

Response: As suggested, the materials are now sorted in alphabetic order.

REVIEWER # 1

Minor Concerns

In step 2.1. Please describe or propose a method for sterilization of surgical instrument and provide a list of what are the instruments

Response: As the reviewer suggested, sterilization methods are decribed in step 2.1, line 128. The list of surgical instruments was included as a supplementary file (JoVE_Materials.xls).

Although there are illustrations of the regions of the brain were the cuts will be done, are not very illustrative. Would be possible to include better illustrations or photos that can indicate better anatomical structures for references?

Response: In the new version of the manuscript we include photos taking at the coronal view of the adult vole brain, illustrating the anatomical structures to be dissected (Figure 1 and Figure 2).

In 6.1, The cells to be cultured are the ones that passaged through the cell strainer or the ones retain in the cell strainer? and clarify which medium should be used?

Response: In this new version, we specify that the cultured cells are those that passed through the strainer and they are seeded at B27 medium (6.1 step, lines 211-212).

In 6.3, the culture conditions should include high humidity?

Response: Thank you for the comment. In this new version, we add in the 6.3 step (line 214) the relative high humidity (90-95%) for culture conditions.

In 6.4, the concentration of growth factors should in the fresh medium should be as indicated in step 6.2, or double? because after mixing with the remaining half of the original medium, those concentrations will drop to half? please clarify

Response: We agree with the reviewer that in the changing medium, the concentration of growth factors is double (line 217).

In 6.5, please clarify: without changing medium? What final concentration of growth factors?

Response: We specified the concentration of growth factors is double for the changing medium (step 6.5, lines 218-219).

Regarding 8.3: describe in a different section how do you prepare the adherent plates.

Response: The procedure to prepare the solutions of Poly-L-Ornithine and Laminin is now included (step 1.7 lines 117-121), as well as how to coat the plates (step 8.1, lines 257-262).

in 8.4.2. differentiation medium is without growth factor supplementation?

Response. We appreciated your comment. The differentiation medium does not contain the growth factor. This information is included in the new version of our manuscript (step now renamed 8.6.2, line 274)

Can you add a section of trouble shooting, identifying possible pitfall and solutions for them

Response: As the reviewer suggested, we now include a paragraph about troubleshooting in the critical protocol steps in the discussion, lines 361-367.

Reviewer #2

Major Concerns

Please include in the introduction at least some of the history and importance of the neurosphere assay as introduced by Reynolds and Weiss (PMID: 1553558). Their work is critical to your success in culturing these cells. Likewise, if other people discovered an important factor in culturing neurospheres that you use, please cite them. Finally, if there are limitations that have been discovered that are important for this paper, can you please cite those in the conclusions?

Response: As the reviewer suggested, literature about the first neurospheres cultures and the importance of growth factors for the culture are now cited (lines 75-80). Also, the limitations of the method are mentioned in the conclusion section (line 370-376).

A strength of the manuscript is the use of prairie voles, but yet you choose a completely different species to represent in Figure 1 where you're taking cells from. Can you please include images of a prairie vole brain and sections? Having used other species, but not the prairie vole, It is unclear to what extent there are differences, which is ultimately their utility-that they are different than mice or rats.

Response: As the reviewer suggested, photos are included of adult prairie vole brain and coronal sections showing the neurogenic structures (Figures 1 and 2).

Please list N for the number of animals the experiment was performed on for statistical analyses.

Response: The number of animals per experiment is provided in the new version of the manuscript in the Figure Legends (Table 1, lines 312-315; Figure 3, line 330 and Figure 4, lines 341-344).

Minor Comments:

Line 77: in vitro should be italicized

Line 80: in vitro should be italicized

Response: As indicated above, all recommendations were made in this new version of the manuscript.

Line 84: it is not clear, perhaps because the grammar is incorrect, by, "the properties of the cells can be sex-dependent".

Response: In this new version, the grammar mistake was corrected. Line 90.

Line 94: What kind of filter???

Response: In this new version of the manuscript, we describe the filters, lines 123-124.

Line 96: Please indicate PBS abbreviation and composition. Line 97: Please state DMEM abbreviation

Response: The meaning of the acronym PBS and DMEM is provided in lines 101 and 106. PBS was bought to Thermo Fisher (10010023).

Line 97-98: These values add up to 505 mL. Shouldn't you use 485 mL of DMEM/F12?

Response: We appreciate the correction. The correct value is now in the manuscript, line 101.

Lines 116: How is completely anesthetized measured? Do you use toe pinch test?

Response: We added in line 136 that we verified the depth of the anesthesia by the pedal reflex absence in response to a firm toe pinch.

Line 131: Please indicate that the blocks are generated by coronal divisions.

Response: As the reviewer suggested, we mention that the blocks are generated by coronal divisions (lines 156-157).

Line 165: Please replace biosecurity with biosafety.

Response: As indicated above, 'biosecurity' was replaced by 'biosafety', line 189.

Line 169: Do you use plastic or glass pipette/tips? It looks to be plastic, but aren't Pasteur pipettes more appropriate? I only ask to make sure that this is correct.

Response: We used 1 mL plastic pipette tips. We do not use Pasteur pipettes for cell culture in the laboratory, so we do not know if they would be more efficient than plastic tips. In the future, we will evaluate the efficiency of Pasteur pipettes.

Line 194: As a general note, usually animal work uses P to denote postnatal day, but here it is used to indicate passage which is slightly confusing.

Response: In this new version of the manuscript, "P0" (passage zero) was replaced for "primary culture" and the abbreviations 'P1' and 'P2' for 'Passage 1' and 'Passage 2' to avoid confusion with postnatal days.

Line 212: It is not clear what is meant by 1 mL cut tips-is this an instrument? (This is found in several other spots.)

Response: 1 mL pipette tips with the end cut-off were used to avoid damaging the spheres when they are collected. We included a note in line 239 to clarify the point.

Line 253: obtain should read obtained

Response: As indicated above, the grammatical mistake was corrected. Line 289.

Line 268: Please remove "However,"

Response: As the reviewer indicated, "However" was removed.

Line 328: Please change, "Although the cell culture may be an artifact, it is a valuable tool that could help resolve discrepancies between studies" to "Cell culture is a valuable tool that could help resolve discrepancies between studies in vivo" or similar.

Response: As indicated above, the paragraph was modified according to the reviewer's suggestion, Line 383-384.

Line 333: This needs minor elaboration. Are you suggesting that neurospheres isolated from the different conditions may behave differently in vitro? Is there data to demonstrate this is possible?

Response: As the reviewer suggested, the original sentence: 'Furthermore, the in vitro culture can be a model to evaluate the properties of neural stem cells in response to behavioral changes' was replaced by 'Furthermore, in vitro culture can be a model for evaluating the molecular mechanisms in neurogenic regions that could be associated with behavioral changes in a social model such as prairie vole', lines 387-389.

We change the phrase because we did not mean that neurospheres behave differently depending on conditions, but instead can be used as an in vitro model to understand neural molecular mechanisms that could be involved in behavioral paradigms in the social vole.

Reviewer #3:

Major Concerns:

Line 322-325: The authors had addressed that the number and diameter of neurospheres derived from both neurogenic regions of females are higher in comparison with males. This could be a functional difference in the female brain niche compared to that in males. However, it is also possible that the isolated cells change due to culture adaptation. I agreed with the second issue that the isolated cells change maybe due to culture adaptation. As for the first issue, more proof is needed. For example, how many male(n=?) and female(n=?) tissues were compared, and how the original tissue ensured that the number of cells was roughly the same, because the number of original cells directly affected the later cell culture.

Response: Although the cells' isolate number was not counted from the original tissue, it has been reported in the literature that the neurogenic zones from adult females have an increase in neural stem cells and progenitors, as well as neurogenesis as compared with males in rodents (Diaz D, et al 2008; Kim J, et al 2009). These data suggests that the differences observed in the size and number of neurospheres depend on a higher number of stem cells/progenitors isolated from the females. Another possibility is that these cells have a higher potential for proliferation. Whatever the interpretation is, the primary culture of neurospheres would be an 'in vitro reflection' of the functional differences in neurogenic niches due to sexual dimorphism.

As the reviewer suggested in the new version of the manuscript the number of voles are included in the Figure Legends (Table 1, lines 312-315; Figure 3, line 330 and Figure 4, lines 341-344).

Minor Concerns:

1. Because the cells come from different individuals who have been treated the same way and cultured at the same time, the differences in cell types and numbers can reflect the differences between the original male and female individuals. So I agreed to compare the differences of cells from male and female sources. In figure 6, the differences in the number of cell background between the male and female sources is so large that the image area needs to be re-selected for comparison, and a table or figure below the figures need to add in which cell counts or fluorescence counts were used to compare the differences between male and female cells.
2. Figures 5 and 7 show a similar background number of cells from male and female sources, but the authors need to add a table or figure below the figures in which cell counts or fluorescence counts were used to compare the differences between male and female cells.

Response: We appreciate the reviewer comment, however as JOVE is a methods-based journal rather than answer a specific scientific question, we only show representative images of immunofluorescences to detected neural markers into the neurospheres. Although we include graphs comparing the number and size of neurospheres, in the discussion, we did not focus on the differences that suggest intrinsic differences due to sexual dimorphism, but rather in the protocol itself as well as its future application in the field of neuroscience research.

We agree with the reviewer and images Figure 7 (previously Figure 6) was modified so that the qualitative comparison was not so contrasting.