

# A steroid naturally occurring in the soft water fungi *Achlya heterosexualis* induces neurogenesis *in vitro* and *in vivo* in rat brain



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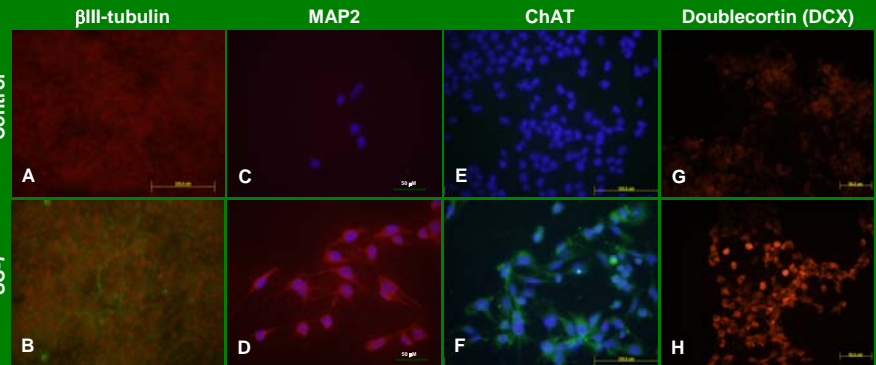
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**ABSTRACT** Repairing brain damage by replacing neuronal loss and restoring the associated functions is certainly the most ambitious and exciting challenge physicians and scientists are currently facing. In that aspect, the concept of stem cell therapy is extremely promising. We previously reported the neuronal differentiation of NT2 cells induced by 22R-hydroxycholesterol, a precursor of steroid synthesis which level is decreased in Alzheimer patient brain. However, precisely because of its steroid precursor properties, 22R-hydroxycholesterol can not be used as a drug. We therefore looked for more "druggable" analogs and we identified a naturally occurring steroid (sc-7) found in the water mold *Achlya heterosexualis* in which it is involved in the seasonal sexual apparatus differentiation. *In vitro*, sc-7 induced neurites sprouting in embryonic mouse teratocarcinoma P19 cells. Two days of treatment resulted in the expression of the neuronal markers MAP2, synaptophysin,  $\beta$ III-tubulin and doublecortin. For the *in vivo* study, adult Long-Evans rats were infused continuously intracerebroventrically with sc-7 over 2 weeks followed by 3 weeks of "maturing" period and received a daily injection of 100 mg/kg BrdU. The compound sc-7 increased the BrdU uptake in the neural stem cells (NSC) present in the ependymal layer and in the subventricular zone as well as in the dentate gyrus of the hippocampus. In both these brain areas, the BrdU uptake co-localized with doublecortin immunostaining, a marker of immature neurons, suggesting an active neurogenesis. In addition cells co-expressing doublecortin, vimentin and labeled for BrdU were also found in the corpus callosum suggesting that the newly generated neurons were migrating from the subventricular area. Although further electrophysiology study is required to assess the functionality of the neurons newly formed, these results suggest that sc-7 might be a good drug candidate to be used in a stem cell therapy strategy.

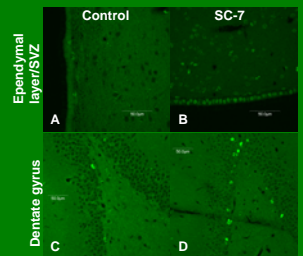
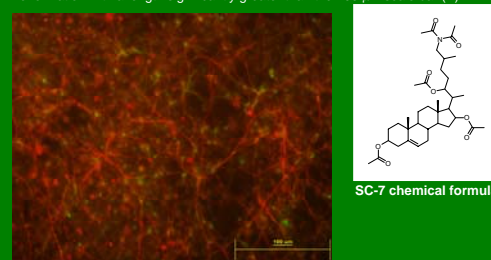
**Introduction** Repairing the brain damages by replacing neuronal losses and restoring the associated functions is certainly the most ambitious and exciting challenge physicians and neuroscientists are currently facing. In that aspect, the concept of stem cell therapy is extremely promising. Hence, the access to the differentiation of stem cells into neurons may serve as a database of specialized cells for regenerative medicine as a treatment for neurodegenerative diseases and brain stroke. We present herein a steroid (SC-7), naturally occurring in the soft water mold *Achlya heterosexualis*, able to trigger *in vitro* the differentiation of neural stem cells into cholinergic neurons and to activate *in vivo* adult rat brain stem cell neurogenesis.

**Cells culture and treatments** Mouse embryonic teratocarcinoma P19 cells were cultured at 37°C in 95% CO<sub>2</sub> in Alpha Minimum Essential medium with ribonucleosides and deoxyribonucleosides containing bovine calf serum (7.5%) and fetal bovine serum (2.5%) on 13 mm diameter glass cover-slip. When cells reached 70% confluence, the medium was replaced fresh medium containing 100  $\mu$ M SC-7. P19 cells were then incubated for 2 days before SC-7 was washed out and replaced by standard medium. The culture medium was changed every 2 days for 5 days or every 2 days for 30 days before cells were fixed for immunocytochemistry.

***In vivo* studies** Male Long-Evans rats weighing 300-325 g (3-4 months old) were housed following a natural day-night cycle with food and water *ad libitum*. Prior to surgery, rats were anesthetized with equitiesn (3 ml/kg, i.p.) and then placed on a stereotaxic frame. Using an electrode micromanipulator, the outlet of an osmotic micropump was implanted into the left cerebral ventricle following the coordinates D = 3.4 mm, L = 1.4 mm and AP = 0.92 mm caudal to bregma. The tank of the osmotic pump was implanted in a subcutaneous pocket in the midscapular area of the back of the rat. SC-7 at 375 mM was perfused by i.c.v. route at 5  $\mu$ l per hour for 2 weeks. Rats were sacrificed 3 weeks after the end of the brain infusion by intracardiac perfusion, first with a washing solution (NaCl 8 g/l, dextrose 4 g/l, sucrose 8 g/l, calcium chloride 0.23 g/l, sodium cacodylate anhydrous 0.25 g/l, in deionized water) and then with fixative cacodylate buffer (sucrose 40 g/l, paraformaldehyde 40 g/l, sodium cacodylate anhydrous 10.72 g/l, in deionized water). Brains were stored in fixative cacodylate buffer for an additional week before being embedded in paraffin. To study the neural stem cells proliferation rats were daily injected with a BrdU solution at 100 mg/kg. The first injection took place the day following the surgery and the last injection was performed the day previous to the euthanasia.

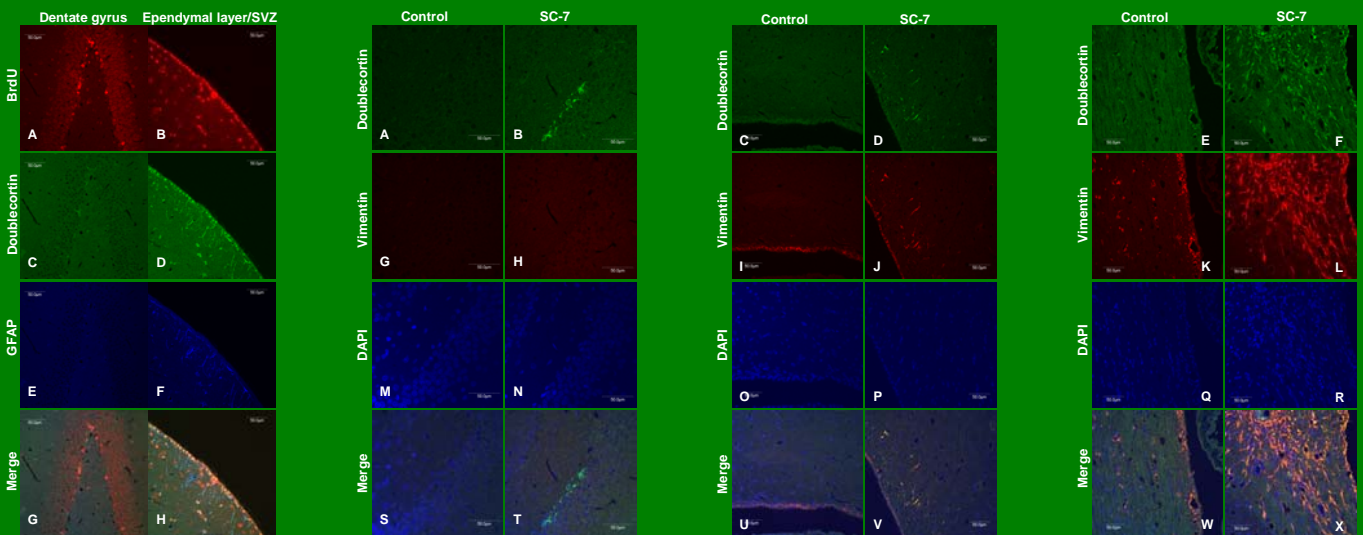


**Identification of neuronal and non-neuronal markers in the differentiated P19 cells** We conducted this experiment to assess the ability of SC-7 to induce the neuronal differentiation of the P19 cells. P19 cells were treated for 2 days followed by a 5 days wash out period. No neuronal marker was observed in controls (A, C, E, G). SC-7-treatment induced a strong expression of the different neuronal markers studied,  $\beta$ III tubulin (B), MAP2 (D) and ChAT (F). The migrating neuroblast marker DCX was also strongly expressed in SC-7-treated P19 cells (H).  $\beta$ III tubulin immunostaining shows axon-like formation with a length significantly greater than the 100  $\mu$ m scale bar (B).



**Persistence of SC-7 differentiating effect** Neuronal marker expression was also studied after 28 days in culture in order to verify whether the expression observed at 5 days was temporary or permanent. At 28 days, the network of axons and dendrites has dramatically extended as shown by the  $\beta$ III tubulin staining (red). The importance of the synaptophysin labeling (green) shows that the newly formed neurons established synaptic connections.

**BrdU immunolabeling in dentate gyrus and subventricular zone** SC-7 i.c.v. infusion increased BrdU uptake by the stem cells present in the SVZ (B) and in the dentate gyrus (D) compared to the control (A and C) suggesting an increase of the NSC proliferation rate in the SC-7-treated rats.



**Co-localization of DCX and GFAP immunostaining in the SVZ but not in the dentate gyrus of rats treated with SC-7** SC-7 induced the expression of the immature neuron marker DCX (D) which co-localized with the astrocytic marker GFAP (F) in the SVZ but not in dentate gyrus (C, E). This suggests that astrocyte-like type-B cells are, at least in part, responsible for the neurogenesis induced by SC-7 in the SVZ.

**Study of the co-localization of the immature neuronal marker DCX and the NSC marker vimentin** SC-7 induced the expression of DCX, a marker of immature neurons often expressed in migrating neuroblasts, in the NSC present in the dentate gyrus (B) and in the SVZ (D). DCX has also been detected in the corpus callosum (F) and since no resident NSC has been described in this brain structure, DCX labeling might be due to the presence of migrating immature neurons. No DCX was detected in the dentate gyrus (A), SVZ (C) and corpus callosum (E) of the control rats. The neural marker vimentin expression was increased in NSC present in the sub-ependymal tissue (J) where it co-localized with DCX (V). Vimentin was also detected in the ependymal cells (U) but this expression did not seem to have been triggered by SC-7 since the ependymal cells in the control rats were equally labeled (I). Vimentin has been previously described to mediate neurite outgrowth. However, no vimentin was found in the dentate gyrus after SC-7 exposure (H). An intense vimentin immunostaining was detected in the corpus callosum of rats infused with SC-7 (L) where it co-localized with DCX (Y), suggesting that some of the immature differentiating neurons migrate from the SVZ through the corpus callosum. DAPI staining confirmed the presence of a larger number of cells in the corpus callosum of SC-7-treated rats (R) compared to control rats (Q).

**CONCLUSION** The naturally occurring steroid SC-7 induced *in vitro* and *in vivo* NSC differentiation into neurons in the dentate gyrus as well as in the SVZ and seemed to trigger the migration of the newly differentiated neurons. These preliminary data justified the interest of SC-7 as a potential small molecule to be used for stem cells therapy based on the *in situ* differentiation of existing NSC.