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Original Article

Specification and Differentiation of Serotonergic Neurons

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Abstract

Serotonin is an important neurotransmitter with multiple functions in the whole central nervous system. Its synthesis, however, is restricted to a very limited number of cells in the brainstem raphe nuclei with a vast axonal network. These cells express markers of the serotonin lineage such as the rate-limiting enzyme in serotonin synthesis, tryptophan hydroxylase 2, the serotonin transporter, and the transcription factor Pet1. Pet1 together with Lmx1b, Nkx2.2, Mash1, Gata2, Gata3, and Phox2b form a transcriptional network, which specifies the differentiation of serotonergic neurons around embryonic day 11 in the mouse. These cells are generated in rhombomeres r1–r3 and r5–r7 caudal to the midbrain–hindbrain organizer under the control of the fibroblast growth factors 4 and 8 and sonic hedgehog (SHH) from precursors, which have produced motoneurons before. Because serotonin is a relevant pathophysiological factor in several neurological diseases such as bipolar disorder and depression tools to generate or maintain serotonergic neurons might be of therapeutic value. Such tools can be assessed in embryonic stem cells, which can be differentiated in vitro to produce serotonergic neurons. Culture systems for these cells including embryoid bodies based and monolayer differentiation have been established, which allows the generation of up to 50% serotonergic neurons in all neurons developed.

Index Entries: Serotonin; Raphe nuclei; development; embryonic stem cells; neurogenesis; embryoid body.

Introduction

Serotonin (5-hydroxytryptamine, [5-HT]) is at the same time a potent neurotransmitter with multiple functions in the central and peripheral nervous system and a hormone mostly released by platelets on activation with essential functions in hemostasis and other nonneuronal processes *(1–3)*. The source of most serotonin in the periphery is the gut, where it is synthesized by enterochromaffin cells of the mucosa, which generates more than 90% of the body's hormone and releases it in the bloodstream, where it is taken up by platelets and transported to all vascularized tissues. Enterochromaffin cells use the TPH1 isoform of tryptophan hydroxylase as first and rate-limiting enzyme in serotonin generation. We recently discovered a second isoform of the enzyme (TPH2) encoded by a distinct gene and expressed nearly exclusively in neurons, where it is the only isoform forming the basis of the central serotonergic system *(2)*. This system consists of only about 20,000 TPH2 positive cells, which form a vast axonal network innervating most other areas in the brain and spinal cord. As a consequence, 5-HT has been shown to be involved in a multitude of central actions such as the control of sleep, mood, and feeding. A very intriguing theory interprets this network as response system to CO_2 overload *(4)*. The serotonergic neurons are located in the raphe nuclei, which form two clusters in the brainstem, a rostral with groups B6–B9 and a caudal with groups B1–B5 innervating the brain and the spinal cord, respectively *(5)*. Both groups show overlapping but also divergent developmental pathways. In the following section the normal development of these serotonergic neurons will be first described and then we list currently available methods to develop such cells from embryonic stem cells.

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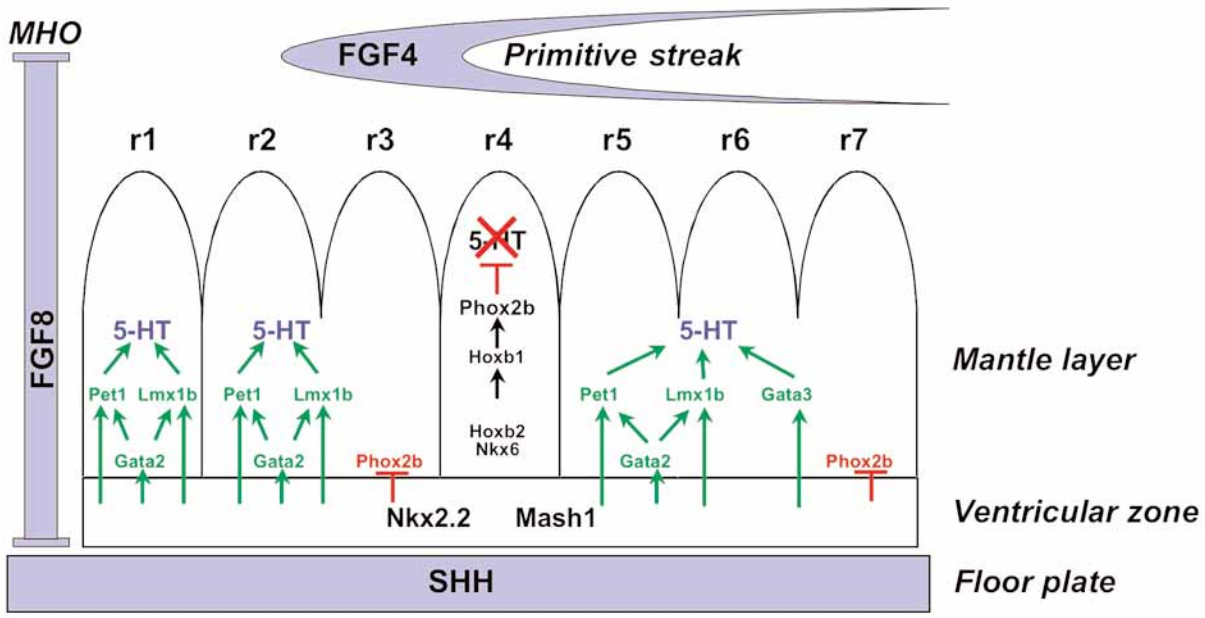

Fig. 1. Induction and differentiation of serotonergic neurons in vivo (see text for explanation). MHO, midbrain–hindbrain organizer; r1–r7, rhombomeres 1–7; SHH, sonic hedgehog; FGF, fibroblast growth factor.

Specification and Differentiation of Serotonergic Neurons In Vivo

The midbrain–hindbrain organizer (MHO) defined by the expression of the transcription factors Otx2 and Gbx2 is a major determinant for the development of serotonergic neurons. They appear caudal of it and dopaminergic neurons are generated rostrally (Fig. 1). When the organizer is moved, the area of one transmitter is increased to the expense of the other *(6)*. One important diffusible factor generated at the MHO is FGF8 *(7)*, which together with FGF4 produced by the primitive streak and SHH synthesized by the floor plate defines the inductive center for serotonergic differentiation *(8)*. In this zone in rhombomeres 1–7 (r1–r7), neural progenitors, which have produced branchiomotor and visceromotor neuronal precursors before, start to differentiate to serotonergic neurons at embryonic day 10.5 (E10.5) in the mouse. Exceptions are r1, which never generates motoneurons, and r4, which carries on producing motoneurons and never gets serotonergic.

The lack of serotonergic differentiation in r4 is owing to the persistence of the expression of Phox2b, an inhibitor of serotonergic differentiation and separates two zones of serotonergic fate with partly distinct mechanisms of differentiation forming the basis for the two clusters of adult serotonergic neurons mentioned earlier *(9)*. In all other rhombomeres (except r1, where it is never expressed), *Phox2b* is switched off at E10.5 probably by Nkx2.2. In conjunction with Mash1 and Nkx2.2 also postmitotically activates the transcription factors Gata3, Gata2, Lmx1b, and Pet1 *(10)*, which together define the serotonergic cell type by activating marker genes such as for TPH2, aromatic amino acid decarboxylase (AAAD), the serotonin transporter (SERT) and the vesicular monoamine transporter (VMAT) 2, and some serotonin receptors *(11)*. Thereby, Gata3 is only essential for the development of the caudal cluster but not the rostral one, and the function of Pet1, which is nevertheless expressed very specifically in all serotonergic neurons and until adulthood, is partially redundant since Pet1-deficient mice still retain about 30% of such cells *(12)*. Thus, only Mash1, Lmx1b, and probably Gata2 are indispensable for all serotonergic neurons to develop properly.

Transcription Factors Involved in Serotonergic Differentiation

Phox2b

Phox2b (paired-like homeodomain protein 2b) is a transcription factor belonging to the Q50 paired-like class *(13)*, which represses serotonergic differentiation in r2–r7. Phox2b-deficient mice lack all visceromotor neuron precursors and serotonergic neurons are extensively produced in r2–r7, including r4 *(9)*, confirming that Phox2b is a central repressor of serotonergic fate (Fig. 1). The formation of serotonin neurons is enabled in r2–r3 and r5–r7 through inhibition of Phox2b by Nkx2.2, whereas in r4, Hoxb1, Nkx6.1, and Nkx6.2 sustain its expression and thereby block serotonergic differentiation *(14)*.

Mash1

Mash1 (mouse achaete-scute homolog 1) is a basic-helix-loop-helix (bHLH) transcription factor, which is already detected in r1–r7 during motor neuron generation but becomes only essential when serotonergic neurons are developed in this zone. Thus, in Mash1-deficient mice no cells expressing the downstream factors Pet1, Lmx1b, Gata2, Gata3, and also no serotonergic neurons appear *(10)*. However, Nkx2.2, Phox2b, and SHH retain their normal pattern of expression in these mice. Furthermore, *Mash1* specifies the serotonergic phenotype in neural crest derivatives like enteric and other peripheral neurons *(15)*.

Nkx2.2

Nkx2.2 (NK transcription factor related, locus 2) is expressed transiently starting at E10.5 in all serotonergic precursors. Mice

lacking this factor do not express Gata3, Lmx1b, and Pet1 in caudal raphe nuclei and no serotonergic neurons develop in this area in contrast to the rostral raphe nuclei, in which all these factors and such neurons persist (9,16,17). Together with Lmx1b and Pet1 it can induce ectopically the development of serotonergic neurons in the chick neural tube (18).

Lmx1b

Lmx1b (LIM homeobox transcription factor 1β) is required for the formation of the entire serotonin system in the hindbrain, because its deletion in mice leads to the absence of such neurons in the brain (17,18). It is expressed in developing serotonergic neurons together with Pet1 starting around E11 in the rostral cluster of serotonergic differentiation and 1 d after in the caudal one consistent with the delayed appearance of serotonergic cells in the latter region (18). Its ablation does not affect the expression of Nkx2.2, Gata3, and SHH and only partly the one of Pet1, putting these factors upstream or in parallel to Lmx1b (17,18). Together with Nkx2.2 and Pet1 it can induce ectopically the development of serotonergic neurons in the chick neural tube (18). In addition, Lmx1b is important for the development of dopaminergic neurons (19).

Pet1

The ETS domain transcription factor Pet1 (pheochromocytoma 12 ETS [E26 transformation-specific]) is a specific marker for all serotonergic neurons from E11 until adulthood (11). Recently, this unique specificity was confirmed by the use of the Pet1 promoter to target marker genes exclusively to 5-HT neurons in transgenic mice (20). Pet1 binding sites are found in the promotor regions of several genes expressed in serotonergic neurons such as AAAD and SERT (11). In mice lacking Pet1, 70% of serotonergic neurons fail to differentiate, whereas in the remaining Pet1-deficient neurons diminished expression of VMAT2, TPH, and SERT was observed (12). These animals survive but show anxiety-like and aggressive behavior.

Gata2 and Gata3

Six Gata (GATA-motif binding) transcription factors exist in vertebrates characterized by C4-type zinc-finger motifs and two of them, Gata2 and Gata3, are expressed in the developing brain (21). Experiments in chicks show that Gata2 is necessary and sufficient for the induction of Lmx1b and Pet1 and serotonergic neurons in r1, but not more caudally (22). In hindbrain explant cultures of Gata2-deficient mice, no 5-HT neurons are developed indicating that Gata3 might be also pivotal for serotonergic differentiation in general. In contrast, Gata3 is not required for the differentiation of the rostral 5-HT neurons (23) and appears unable to substitute for the loss of Gata2 in r1. However, in Gata3-deficient mice, around 80% of serotonergic neurons in the caudal clusters and 30% in the rostral clusters are missing (10). Nevertheless, the expression of Pet1 and Lmx1b was unchanged in Gata3 knockout mice showing that these factors act in parallel.

Differentiation of Serotonergic Neurons From Embryonic Stem Cells In Vitro

Mouse ES cells are pluripotent cells, originating from mouse blastocysts and capable to differentiate into cellular derivatives of all three primary germ layers (24–26). In the meantime, differentiation methods were also developed and optimized for human and primate ES cells. Therefore, ES cells represent an excellent model for the discovery of new genes and factors involved in the differentiation of certain cell types, as well as for cell-based transplantation therapy. The pioneering work to generate ES cells from in vitro cultured blastocysts produced by nuclear transfer technology (27–29) and to reprogram somatic cell nuclei in hybrid ES cells (30) opens new perspectives in cell therapy and promises to solve the problem of immune-tolerance after stem-cell transplantation.

The targeted differentiation of ES cells into populations of cells enriched in neurons, which were also shown to be functionally active, was established 10 yr ago (31–33). It was shown that retinoic acid (RA) dramatically increases the number of neurons in relation to other cell types and represses mesodermal gene expression (34,35). Nevertheless, with standard methods employing RA induction only very few 5-HT neurons (around 2% or less) can be produced. Additionally, RA-free protocols were developed, including coculture systems with bone marrow-derived stromal cells (36), VIP and PACAP induction (VIP and PACAP are potent regulators of the proliferation and patterning of neural tube [37]), and methods established in the McKay lab, which includes selection and amplification of neural precursor cells with defined serum-free media (38). Most of these studies were conducted to optimize the conditions for ES cells to become dopaminergic neurons, which are of special importance for the treatment of Parkinson's disease (39–41). In contrast, the ability of ES cells to differentiate to the serotonergic fate was not thoroughly studied to date. Most of serotonergic differentiation protocols use as a basis optimized dopaminergic differentiation conditions. As the pathways of differentiation in vivo are similar between the two neuronal types (42), 5-HT neurons are always enriched after dopaminergic differentiation, and the serotonergic fate can be further promoted with FGF4. Besides ES cells also neural stem cells (NSC), that can be isolated from both developing and adult mammalian brain provide exciting perspectives for in vitro analysis of early nervous system development and the generation of donor cells for neural repair. The differentiation of NSC and of primary cells from embryonic rat and mouse raphe region to 5-HT neuronal phenotype was intensively studied and used as a model for the optimization of culture conditions. The survival and maturation of serotonergic neurons in a fully defined serum-free culture system of E14 rostral and caudal raphe neurons was shown to be promoted in vitro by a variety of factors, including serotonin itself, BDNF, cAMP, FGFs, and transforming growth factor (TGF)-β (43–46). Furthermore, BDNF was also shown to increase the amount of 5-HT neurons and enhance the number of dendrites in primary culture from the E13 mouse raphe (47). Such factors, which support serotonergic differentiation in primary culture, might also be useful for the ES cell system.

To date, there are four basic methods to generate dopaminergic and serotonergic neurons from ES cells (Fig. 2).

1. Embryoid body formation (Fig. 2, green);
2. Embryoid body formation including selection and amplification of neural precursor cells (Fig. 2, red);
3. Coculture of ES cells with a stromal feeder cell line (Fig. 2, violet); and
4. Differentiation of ES cells in monolayer culture (Fig. 2, blue).

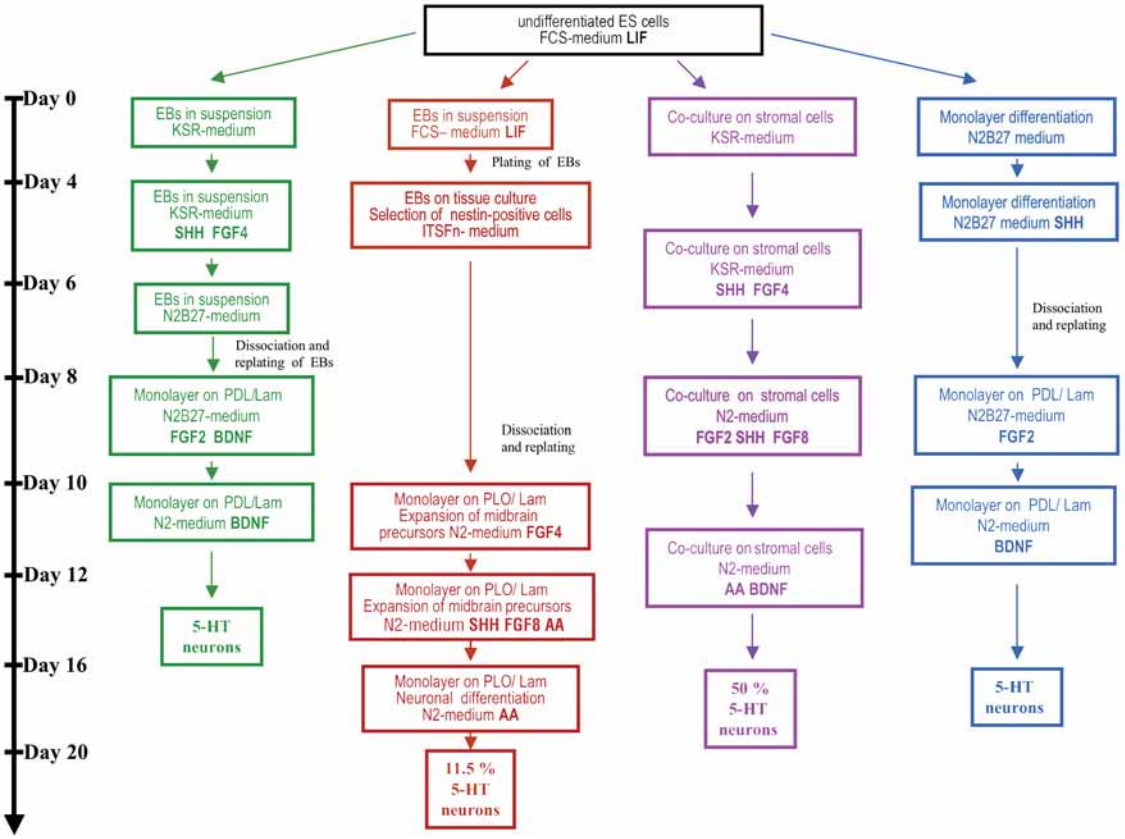

Fig. 2. Methods for in vitro differentiation of mouse ES cells into serotonergic neurons (see text for explanation). EBs, embryoid bodies; FCS, fetal calf serum; LIF, leukemia inhibitory factor; KSR, knockout serum replacement; SHH, sonic hedgehog; FGF, fibroblast growth factor; BDNF, brain-derived neurotrophic factor; PDL, poly-D-lysine; Lam, laminin; PLO, poly-L-ornithine; AA, ascorbic acid; N2-medium, DMEM/Nutrient mixture F12 medium supplemented with N2 additive; N2B27, mixture 1 : 1 N2 medium and B27 medium (Neurobasal medium supplemented with B27 additive); ITSFn-medium, insulin-transferrin-selenium-fibronectin–medium. The scheme summarizes data described by several authors *(38,50,56)* and our own unpublished results.

All these methods are based on the initial culture of ES cells with leukemia inhibitory factor (LIF) and fetal calf serum (FCS) and the omission of serum and LIF already at a very early stage of differentiation (with exception of McKay protocol, in which the initial formation of EBs appears in the presence of serum). The removal of serum partially blocks the differentiation of ES cells into mesoderm and endoderm *(48)*, whereas treatment with SHH and growth factors (e.g., FGF8, FGF4, and FGF2) at later stages drives the cells to midbrain neuronal fate. Terminal differentiation of postmitotic neurons appears in the presence of survival promoting factors either in N2 or N2B27, serum-free media supporting efficient differentiation and maintenance of neural cells in culture *(49,50)*. All four protocols were originally invented for the generation of dopaminergic neurons, but have also been optimized for serotonergic differentiation with maximum rates of 50% of 5-HT-positive neurons in the whole neuronal population.

Embryoid Body Formation

To date, the most frequently applied mode of ES cell differentiation into different cell lineages is the formation in suspension of multicellular aggregates, called embryoid bodies (EBs), that were shown to be enriched in neural cells in the absence of serum *(51,52)*. The method includes, first formation of EBs in nonadherent plastic dishes under serum-free conditions (knockout serum replacement [KSR]) medium, followed by dissociation and replating of cells on poly-D-lysine/laminin (PDL/Lam) 8 d after EB formation was initiated (Fig. 2, green). After replating neural precursor cells are first enriched for 2 d by the addition of FGF2, which is a strong mitogen and then terminal differentiation of neurons is completed after withdrawal of this growth factor. Any kind of classical RA treatment of EBs leads to nearly pure neuronal populations, but with a very rare appearance of 5-HT neurons. The exchange of RA by SHH-induction increases several fold the quantity of serotonin neurons, whereas the purity of the neuronal population remains unchanged (Fig. 3).

McKay Protocol

Based on previous findings *(53)* an efficient five-stage protocol, that combine methods of ES-cells differentiation with a methodology for the differentiation of NSC was developed (traditionally called McKay or five-stage protocol *[38]*). The first stage includes, expansion of undifferentiated ES cells in the presence of FCS and LIF (stage 1; Fig. 2, red), then differentiation is initiated by formation of EBs in serum-containing medium

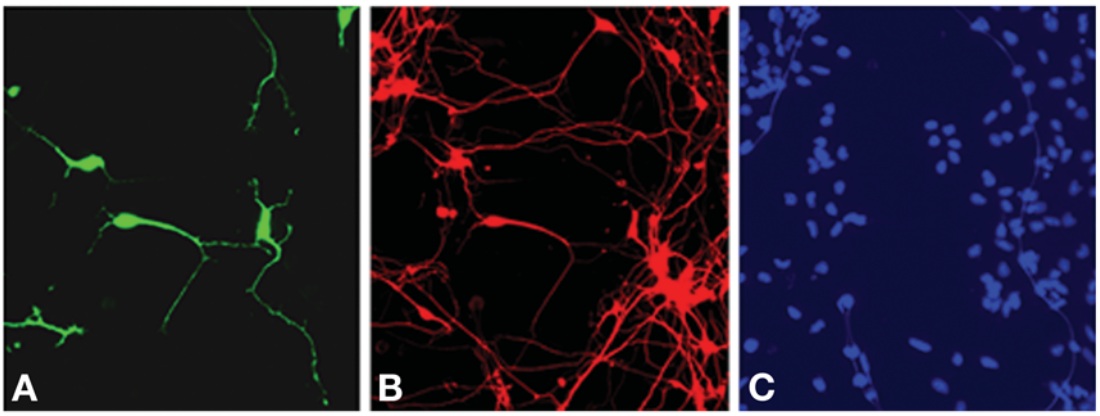

Fig. 3. In vitro differentiation of Es cells to serotonergic neurons. ES cells were differentiated using the EB-protocol with SHH-induction at day 6 of EB formation. **(A)** Staining for 5-HT (green); **(B)** staining for panneuronal marker Tuj I (red); **(C)** DAPI-staining (blue) for nuclei.

(stage 2) resulting in aggregates made up of cells of all three primary germ layers. After 4 d, EBs are plated into tissue culture plastic dishes (stage 3) and nestin positive neuroectodermal cells migrating out of EBs are selected in insulin-transferrin-selenium-fibronectin (ITSFn) medium, whereas nonneural cells are partially eliminated as they hardly survive serum-free conditions. After 6–8 d of selection, cells are dissociated, migrated cells are mechanically separated from rest-aggregates containing meso- and endodermal cells, and almost pure neural precursor cultures are replated to poly-L-ornithine/laminin (PLO/Lam). These neural precursors are expanded in N2 medium for 4–6 d in the presence of mitogens and inductive factors (stage 4). Terminal differentiation and maintenance of functional neurons appears after withdrawal of growth factors in the presence of survival-promoting factors, such as ascorbic acid (AA) (stage 5). The basic protocol does not allow effective differentiation of 5-HT neurons, since it was optimized to drive the dopaminergic fate. However, addition of SHH and FGF4 during stage 4 was shown to increase the population of serotonergic neurons up to 11.5% *(54)*. Surprisingly, when the basic protocol was used for the differentiation of rhesus monkey ES cells, nearly pure cultures of serotonergic neurons were produced even without the application of specific differentiation factors, such as SHH, FGF4, and FGF8 *(55)*.

Coculture on Stromal Cell Feeder Layer

The method is based on the feature of some mouse stromal cell lines to promote neural differentiation. First stromal cell-derived inducing activity (SDIA) was identified by coculture of mouse ES-cells with the PA6 cell line *(36)*. Later studies revealed that other bone marrow-derived stromal feeder cell lines (MS5 and S17), as well as primary stromal feeder cells obtained from the aorta-gonad-mesonephros promote efficient neural differentiation *(56)*. The neurons were shown to be functional and could be used for injury repair in mice. The MS5 line is to date the most effective in combination with SHH and FGF4 to induce serotonergic differentiation (up to 50% 5-HT positive cells in all neurons obtained; *see* Fig. 2, violet). The great advantage of the method is that during differentiation cells are amplified more then 1000 times (each ES cell gives rise in average to 2000 neurons *[56]*), whereas using the most advanced EB-protocol, which includes propagation of neural precursors, only seven neurons can be generated from one ES cell *(38)*.

Monolayer Differentiation

An elegant method to differentiate ES cells in adherent monoculture was developed using a Sox1-GFP-puro knockin reporter ES cell line *(50)*. Sox1 is the earliest marker of neuroectoderm and such a cell line allows to visualize the differentiation procedure and to select neural precursor cells either by application of selective antibiotics (puromycin) or by fluorescence-activated cell sorting *(57)*. The monolayer is initiated by plating ES cells on gelatin-coated dishes in serum-free N2B27-medium, which was optimized for this protocol. Further steps are quite similar to the standard EB-method and comprise replating of cells after 6–8 d of monolayer differentation to PDL/Lam tissue culture dishes in the presence of FGF2 and terminal differentiation of neurons after its withdrawal. The method is not easily reproducible since the ES cells are very sensitive to the serum-free conditions during the monolayer culture. The addition of SHH from day 4 to 6 of differentiation, followed by replating of cells results in a percentage of 5-HT neurons similar to the EB-protocol (Fig. 2, blue).

Perspectives

Several methods have been established to produce serotonergic neurons from differentiating ES cells. Still, these methods have to be optimized and the application of knowledge stemming from the increasing insights into the embryonic development of 5-HT neurons should be helpful for this purpose. Possible strategies include the overexpression of known transcription factors such as Pet1, Gata2, Gata3, and Lmx1b, involved in the serotonergic cascade as well as driving serotonergic differentiation by CO_2-treatment, which might be a key mediator in this process *(58)*. Another possibility would be to interfere with pathways leading to the differentiation of other neuronal subtypes originating from the same brain area. Most promising candidates are blockers of dopaminergic differentiation and inhibitors of Phox2b. In vitro differentiated serotonergic neurons will be useful to further characterize the interactions of extrinsic factors and transcriptional cascades acting in vivo to create this neuronal subtype. Furthermore,

such cells themselves or the methods to create or maintain them in culture can be used for the replacement of 5-HT neurons in psychiatric and neurodegenerative diseases with a loss in serotonergic tone.

Abbreviations

5-HT	Serotonin (5-hydroxytryptamine)
AA	Ascorbic acid
AAAD	Aromatic amino acid decarboxylase
AGM	Aorta-gonad-mesonephros
BDNF	Brain-derived neurotrophic factor
bHLH	Basic-helix-loop-helix
E14	Embryonic day 14
EBs	Embryoid bodies
ES cells	Embryonic stem cells
FCS	Fetal calf serum
FGF	Fibroblast growth factor
Gata	GATA-motif binding
ITSFn	Insulin-transferrin-selenium-fibronectin-medium
KSR	Knockout serum replacement
LIF	Leukemia inhibitory factor
Lmx1b	LIM homeobox transcription factor 1β
Mash1	Mouse achaete-scute homolog 1
MHO	Midbrain–hindbrain organizer
Nkx2.2	NK transcription factor related, locus 2
NSCs	Neural stem cells
PDL/Lam	Poly-D-lysine/laminin
Pet1	Pheochromocytoma 12 ETS
Phox2b	Paired-like homeodomain protein 2b
PLO/Lam	Poly-L-ornithine/laminin
SDIA	Stromal cell-derived inducing activity
RA	Retinoic acid
SERT	Serotonin transporter
SHH	Sonic hedgehog
TGF-β	Transforming growth factor-β
TPH	Tryptophan hydroxylase
VMAT2	Vesicular monoamine transporter 2

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