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Expression of serotonin receptor subtypes in rat brain and astrocyte cell cultures: an age- and tissue-dependent process

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Abbreviations:
bp – base pairs;
CNS – central nervous system;
MASSA – melatonin agonist and selective serotonin antagonist.

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Abstract

Background and Purpose: *Serotonin (5-HT) mediates its effects on neuronal and non-neuronal cells in rat brain through thirteen receptor subtypes. To obtain better insight into 5-HT responsive cells, we investigated the expression of all known rat 5-HT receptors in various brain regions, at various animal ages, and in various sample types.*

Materials and Methods: *The presence of all 5-HT receptor mRNAs was studied with the reverse transcriptase-polymerase chain reaction (RT-PCR) in eight sample groups. We isolated brain cortex and cerebellum from 3-day-old and adult Wistar rats of both sexes, which were used directly for the experiment and for growing the astrocyte cell cultures. We performed the experiment on the astrocyte cell cultures after 3 weeks of growth in culture.*

Results and Conclusions: *We determined that neonatal brain tissue and astrocyte cultures from neonatal rats express more 5-HT receptor subtypes than their adult counterparts. In all tissue samples we detected the same or a larger number of 5-HT receptor subtypes than in the corresponding astrocyte cultures. The expression pattern of 5-HT receptor subtypes also differed between rat cortex and cerebellum. Only subtypes 5-HT$_{1B}$ and 5-HT$_{2A}$ were found in all the samples studied, and we did not detect the message for subtype 5-HT$_3$ in any of the samples. In conclusion, the expression of 5-HT receptors is age- and brain region-dependent, and astrocyte cultures do not reflect the situation* in vivo.

INTRODUCTION

Serotonin or 5-hydroxytriptamine (5-HT) is an important neurotransmitter and neuromodulator in the central nervous system (CNS), but it also affects several peripheral tissues *(1, 2)*. 5-HT influences neuronal growth, survival, and maturation of the developing serotonergic and other neurotransmitter systems in various mammal species as reviewed elsewhere *(2–5)*. In adult life, 5-HT affects the neuronal activity of the glutamatergic and cholinergic system and is involved in the processes of cognition, food intake, mood, sleeping, sexual functioning, pain sensation, and thermoregulation *(2, 6)*. The serotonergic system also plays an important role in the etiopathogenesis of various psychiatric disorders and represents the target for their treatment *(1, 2, 6–8)*. The expression of the 5-HT$_{1A}$ receptor during brain development is essential for anxiety-free behavior in adult mice *(9)*.

Many different effects of 5-HT are the result of its action via at least 15 different 5-HT receptor subtypes in humans and 13 subtypes in rats. The 5-HT receptor family is comprised of seven receptor classes, each with its own structural, transductional, and functional properties. Nevertheless, there are more targets in the 5-HT system, which could give rise to the development of new serotonergic drugs. To make this a reality, better knowledge about 5-HT receptors is a necessary starting point.

Astrocytes are macroglial cells that are responsible for the development and maintenance of nerve cells and their synapses. Their ratio to nerve cells is age- and brain region-dependent. Astrocytes bear receptors for various neurotransmitters, including 5-HT. The stimulation of astrocyte 5-HT receptors renders changes in the intracellular cyclic adenosine monophosphate (cAMP) and nerve-growth factor (NGF) concentration, again in an age- and brain region-dependent manner *(10)*. Hirst *et al. (11)* found messenger ribonucleic acid (mRNA) for all G protein-coupled 5-HT receptor subtypes in astrocyte cultures from newborn rats, except for 5-HT_4 and 5-HT_{5A}; they did not study the expression of the 5-HT_3 receptor. Aside from this, not much has been published about the expression of 5-HT receptors and their functionality in astrocytes.

This study examined the expression pattern of all known 5-HT receptor subtypes in astrocyte cultures from cerebellum and cortex of newborn and adult Wistar rats. We compared the results with corresponding expression patterns in brain homogenates from the same tissues and at the same animal age. Better knowledge of the 5-HT receptors in astrocytes promises to increase the chances of influencing astrocyte functions with serotonergic drugs.

MATERIALS AND METHODS

Materials

The reagents used in the cell culture experiments were purchased from Life Technologies, Paisley, Scotland, UK, except for fetal bovine serum, which was obtained from Lonza, Verviers, Belgium. We used Ethanol absolute GR for analysis (Merck, Darmstadt, Germany) where appropriate. The origins of all other materials are listed in the appropriate subsections.

Animals

The study was carried out using neonatal (3-day-old) and adult (at least 3 months old and weighing 180–200 g) Wistar rats of both sexes. We did not divide our experiments according to animal gender because we did not expect to find any qualitative differences in 5-HT expression. In the literature, all of the studies dealing with this topic use quantitative approaches for evaluating 5-HT expression *(12, 13)* or for measuring 5-HT receptor protein immunoreactivity *(12–14)*, and all gender-related differences are minimal. Thus, in our opinion, there should be no qualitative differences in 5-HT receptor expression between male and female rats, which was also shown in our preliminary studies.

Rats were housed four to five per cage, the room temperature was kept at 23 ± 1 °C, and food and water were available ad libitum. All of the animals received care in compliance with the European Convention on Animal Care and were used under the protocol approved by the Veterinary Administration of the Republic of Slovenia with regard to the care and use of laboratory animals (number: 323-02-151/2005/2).

Tissue removal

The rats were decapitated and the whole brain was removed from their heads. Cerebellum and cortex were isolated from the brain tissue. Immediately after isolation, all tissues were fresh-frozen at -70 °C and used for further procedures.

Astrocyte cell cultures

Astrocyte cell cultures were prepared from the cerebella and cortices of neonatal and adult rats of both sexes, and cultured as previously described *(15)* with one modification. After removal of the meninges, the tissue was washed and aspirated in Leibowitz L-15 medium, 10 times using a 10 ml pipette and 10 times using a 5 ml pipette. The tissue was then taken up three times into a syringe with a 25, 22, and 20G needle, respectively, after which it was passed through a 100 µm cell strainer (Falcon, Oxnard, Canada). After pelleting, the cells were plated in high-glucose Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum, 1 mM pyruvate, 2 mM glutamine, 25 mM glucose, and 25 µg/ml gentamycin in 95% air-5% CO_2. Confluent cultures were shaken at 225 rpm overnight, and the medium was changed the next morning; this was repeated a total of three times. After the third overnight shaking, the cells were trypsinized and cultured for 24 h in 10 µM cytosine arabinoside. After reaching confluence again, the cells were subcultured into 100 mm sterile Petri dishes.

The purity of the cultures was assessed with immunocytochemical analysis, which revealed 98% glial fibrillary acidic protein (GFAP) positive cells (type-1 astrocytes) and 2% OX42 positive cells (microglia).

RNA isolation from tissues

The total ribonucleic acid (RNA) isolation from tissue samples was performed with RNAgents® Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's protocol. The quantity and purity of isolated total RNA was estimated by measuring the absorbance at 260 and 280 nm with a Perkin-Elmer MBA 2000 spectrophotometer.

RNA isolation from cell cultures

The total RNA isolation from cell cultures was performed with the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's protocol. The quantity and purity of isolated total

TABLE 1

Primer pair sequences and corresponding PCR product size lengths for all 5-HT receptors studied and for cyclophilin, which was used as a control.

Target mRNA	Sense (downstream, forward, left) primer	Antisense (upstream, reverse, right) primer	Product size (base pairs)
5-HT$_{1A}$	CTCCTTGGCGGTTACTGAT	GTAGATGGTGTAGCCGTGGT	367
5-HT$_{1B}$	TGATGCGGTGGACTATTCTG	ACCGTGGAGTAGACCGTGTAGA	186
5-HT$_{1D}$	GCATCTCTGTGTCATCGCTCT	ATGTGTTCACCAGGCAGTCA	197
5-HT$_{1F}$	TCCATCTTGCACCTGTCGGC	AACCAACATATTACAAATGCGCCC	593
5-HT$_{2A}$	GGCATCGTGTTCTTCCTGTT	TCAGCATCTTCCTGTGAGTTCT	335
5-HT$_{2B}$	CTCCGAAGTTCAACCATTCAGTC	TTGCTTGCGTCCTCCTCATC	157
5-HT$_{2C}$	TCCTTCGTGGCATTCTTC	ACACACATAGCCAATCCACA	429
5-HT$_{3}$	ATGGCTCTGCTGGTGATAA	TCAGTCTTGTTGGCTTGG	191
5-HT$_{4}$	GGAACAACATCGGCATAGT	TGATATAGCCAAGCCAGAGG	429
5-HT$_{5A}$	ATGGCTATCTCGGATGTGCTA	CGACTGACCTGGCATTCTTC	344
5-HT$_{5B}$	GACTCCTTGACATAGCCTCTC	TACTGAGCCATCTTGACGAC	170
5-HT$_{6}$	CTGTACCTGCAGTCACCATA	CATACAGCGCGTTCAGCAT	316
5-HT$_{7}$	AATCATTGGCTGAGACTGC	CACTCTTGTGGATGTGGACT	278
cyclophilin	TGGACCAAACACAAATGGTT	TGATCTTCTTGCTGGTCTT	161

RNA was estimated by measuring the absorbance at 260 and 280 nm with a Perkin-Elmer MBA 2000 spectrophotometer.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Equal amounts (250 ng per reaction) of total RNA were used for RT-PCR, which was performed with the GeneAmp® Thermostable rTth Reverse Transcriptase RNA PCR Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. The reactions took place in the Perkin-Elmer GenAmp System 2400 with the following temperature profile:

a) Reverse transcription	15 min	70 °C
b) Denaturation	1 min	95 °C
c) Melting	10 s	95 °C
d) Annealing & extending	15 s	60 °C
e) Elongation	7 min	60 °C

Steps c and d were repeated in cycles for a total of 35 times.

We used a mixture of deoxyribonucleotide triphosphates Genamp dNTP mix, manufactured by Applied Biosystems, Foster City, CA, USA. Primers were designed with the computer software Primer 3 (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) and synthesized at Applied Biosystems, Foster City, CA, USA. Cyclophilin gene was used as a positive control and samples without a reverse transcription step as a negative control. The primer sequences and corresponding product sizes are listed in Table 1.

DNA electrophoresis

The agarose gel electrophoresis was performed with the Sub-Cell GT Agarose Gel Electrophoresis System (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. The 2% agarose gel was prepared with 2 g of Agarose LE analytical grade (Promega, Madison, WI, USA) and 100 ml of 1× Tris-Borate-EDTA buffer, and 2 µl of 10 mg/ml ethidium bromide (Bio-Rad, Hercules, CA, USA) was added. 1× Tris-Borate-EDTA buffer was prepared by diluting the Tris-Borate-EDTA buffer 10× Concentrate (Sigma, St. Louis, MO, USA). Products on the gel were stained with Blue/Orange 6× Loading Dye (Promega, Madison, WI, USA) and visualized at 302 or 366 nm. As a size marker, the 100 bp DNA Ladder (Promega, Madison, WI, USA) was used.

RESULTS

Axons from serotonergic neurons in the raphe nuclei terminate and exert their action in the cortex, cerebellum, and striatum by stimulating 13 different 5-HT receptor subtypes in rats. The mRNA expression of 13 different 5-HT receptor subtypes in rat brains and in astrocyte cultures was a process dependent on brain region, animal age, and possibly cell type.

The expression of 5-HT receptor subtypes in neonatal rat cerebellum

As shown in Figure 1a, neonatal rat cerebellum showed a message for 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1F}$, 5-HT$_{2A}$, 5-HT$_{2C}$, 5-HT$_{4}$, 5-HT$_{5A}$, and 5-HT$_{5B}$ receptor subtypes, whereas in astrocyte cultures (Figure 1b) prepared from

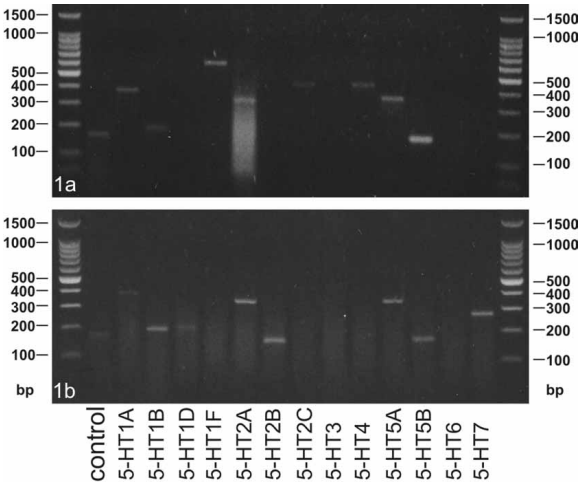

Figure 1. *Neonatal rat cerebellum homogenates (a) and astrocyte cultures (b): Electrophoresis gel photograph stained with ethidium bromide showing RT-PCR products of various 5-HT receptor mRNAs. The corresponding product sizes in base pairs (bp) are shown in Table 1. The housekeeping gene cyclophilin was used as a control.*

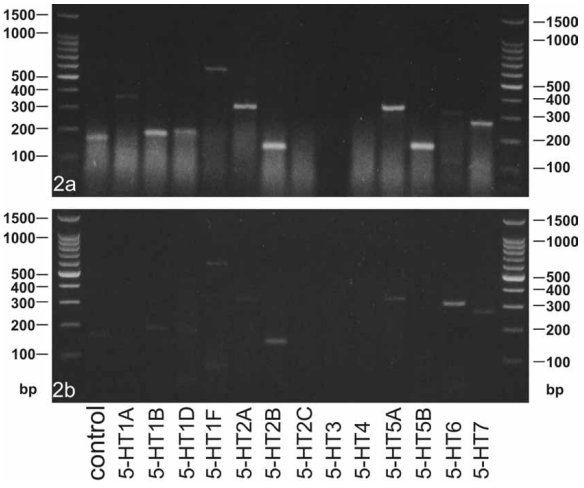

Figure 2. *Neonatal rat cortex homogenates (a) and astrocyte cultures (b): Electrophoresis gel photograph stained with ethidium bromide showing RT-PCR products of various 5-HT receptor mRNAs. The corresponding product sizes in base pairs (bp) are shown in Table 1. The housekeeping gene cyclophilin was used as a control.*

the same brain region of 3-day-old rats and cultured for at least 21 days we could not detect any message for 5-HT_{1F}, 5-HT_{2C}, and 5-HT_4 subtypes, which were present in neonatal rat cerebellar tissue. In neonatal rat cerebellar astrocyte cultures, the expression of 5-HT_{1D}, 5-HT_{2B}, and 5-HT_7 receptor subtypes appeared.

The expression of 5-HT receptor subtypes in neonatal rat cortex

Figure 2 shows the expression pattern of 5-HT receptors in neonatal rat cortex and astrocytes prepared from the same brain region. Neonatal rat cortical homogenates (Figure 2a) expressed mRNAs for 10 out of 13 5-HT receptor subtypes: 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{5A}, 5-HT_{5B}, 5-HT_6, and 5-HT_7; whereas cultured neonatal rat cortical astrocytes (Figure 2b) expressed only seven 5-HT receptor subtypes. They lacked the expression of 5-HT_{1A}, 5-HT_{1D}, and 5-HT_{5B} receptor subtypes, whereas the expression of other subtypes appears to have been retained during culturing.

The expression of 5-HT receptor subtypes in adult rat cerebellum

As shown in Figure 3a, adult rat cerebellum showed a message for 5-HT_{1B}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_{5A}, and 5-HT_{5B} receptor subtypes, whereas in astrocyte cultures (Figure 3b) prepared from the same brain region of normal adult rats and cultured for at least 30 days we could not detect any message for 5-HT_{1F}, 5-HT_{2C}, and 5-HT_{5B} subtypes, which were present in adult rat cerebellar tissue. However, we found the expression of 5-HT_{2B}, 5-HT_6, and 5-HT_7 receptor subtypes, which were present only in cultured astrocytes but not in the corresponding brain tissue.

The expression of 5-HT receptor subtypes in adult rat cortex

Figure 4a demonstrates that adult rat cortical tissue showed a message for 5-HT_{1B}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_4, 5-HT_{5B}, and 5-HT_6 receptor subtypes, whereas in astrocyte cultures (Figure 4b) prepared from the same brain region of normal adult rats and cultured for at least 30 days we detected a message for only 5-HT_{1B} as well as for 5-HT_{2A} and 5-HT_{2B} receptor subtypes.

DISCUSSION

The literature contains many studies dealing with 5-HT receptors. However, comparing their results is difficult because many different methodological approaches and sample types were used in these studies. When interpreting their results, three important questions regarding the study should be answered: what (receptor mRNA or protein), where (brain region, subcellular localization), and when (newborn or adult animals). In some instances, perhaps animal gender and strain could also be a clue to understanding the differing results.

This study addressed the following questions: 1) Is the expression of 5-HT receptors an age-dependent process, 2) Is the expression of 5-HT receptors dependent on brain region, and 3) Do astrocytes cultured from the same brain region and from animals of the same age share the expression of 5-HT receptors with the corresponding brain region?

General observations

Out of all 13 5-HT receptor subtypes studied, only 5-HT_{1B} and 5-HT_{2A} receptors were expressed in all of the brain regions and astrocyte cultures studied. The ubiquitous expression of these two subtypes was expected due to their involvement in numerous physiological and pathological events, and agrees with previous studies *(1, 2, 6, 16, 17)*.

The 5-HT_{5A} receptor was also found in all tissues and astrocyte cultures from all brain regions studied except in adult rat cortex. This agrees with previous findings by Geurts *et al*. (18), who found that 5-HT_{5A} receptor is distributed mainly in rat cerebellum. The precise role of 5-HT_{5A} receptor has not yet been explained, but it seems to be involved in cognitive functions, affective disorders, and brain development *(19)*, and so its wide distribution is not surprising.

The subtype 5-HT_3 was not expressed in any of the samples studied, although there are reports of their expression in rat cortex *(20)*. This discrepancy could be due to two reasons. One may simply be the amount of mRNA below the detection limit of our method. The other reason might be reduced primer specificity. The native 5-HT_3 receptor is an ion channel and is composed of various subunits, and their distribution varies in different tissues. However, the primers are designed according to one subunit only and, if this one is in the minority, the specificity of the primer pair is reduced.

Differences in the 5-HT receptor expression pattern between neonatal and adult rats

During brain development and maturation, 5-HT is not only a neurotransmitter, but also exerts neurotrophic properties by acting on 5-HT_{1A} receptors *(21)*. The replication and regeneration of astrocytes and neurons is much faster at this stage than in adult life. Because of the higher metabolic and synthetic rate, we expected to find more receptors in samples from neonatal animals than in their adult counterparts. In fact this was true in all of the samples studied.

When searching for the receptor subtype that is specific for a particular life period, we realized that the subtypes 5-HT_{1A} and 5-HT_{1D} are expressed only in samples derived from newborn animals. This is consistent with other findings in rodents and further confirms their role in CNS development *(21–24)*.

However, we did not find any 5-HT receptor subtype specific only to adult animals, so we presume that all of the subtypes present in adult animals are already expressed from the developmental phase onwards.

Of special interest is the expression of receptor subtype 5-HT_4, whose message was present in adult cortex, but not in neonatal cortex. However, in cerebellum the 5-HT_4 receptor was expressed only in neonatal tissue, but not in adult tissue. At first glance, these results disagree with the study on 5-HT_4 receptors by Gerald *et al. (25)*, who found this subtype throughout the rat brain. However, more precise comparison reveals that they studied the presence of 5-HT_4 receptor mRNA in adult male CD rats and that the 5-HT_4 mRNA in cerebellum was barely detectable. This nicely demonstrates the above statement about the many important aspects that must be considered when interpreting and comparing results on 5-HT receptor expression: method, animal age, gender, and strain.

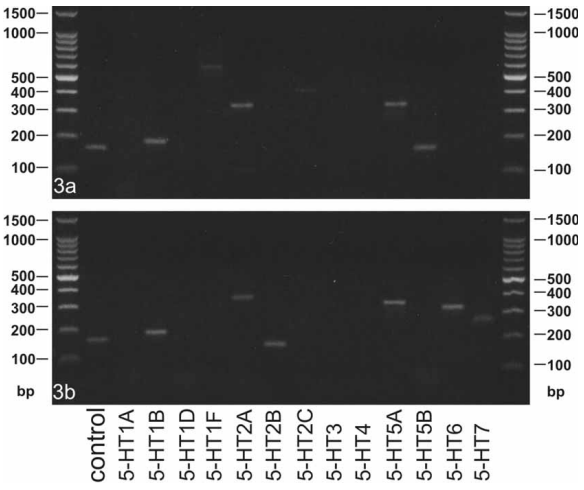

Figure 3. *Adult rat cerebellum homogenates (a) and astrocyte cultures (b): Electrophoresis gel photograph stained with ethidium bromide showing RT-PCR products of various 5-HT receptor mRNAs. The corresponding product sizes in base pairs (bp) are shown in Table 1. The housekeeping gene cyclophilin was used as a control.*

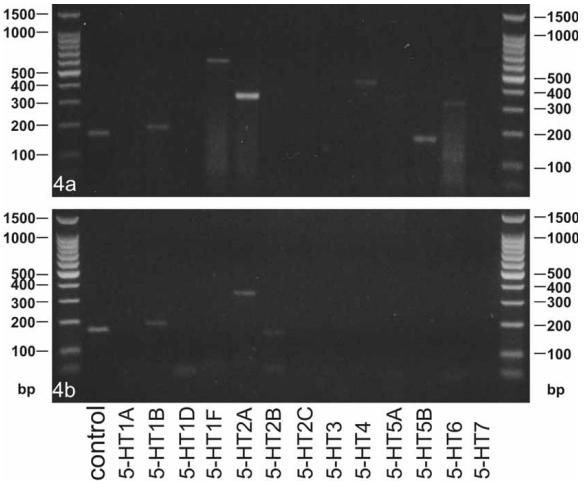

Figure 4. *Adult rat cortex homogenates (a) and astrocyte cultures (b): Electrophoresis gel photograph stained with ethidium bromide showing RT-PCR products of various 5-HT receptor mRNAs. The corresponding product sizes in base pairs (bp) are shown in Table 1. The housekeeping gene cyclophilin was used as a control.*

Differences in the 5-HT receptor expression pattern between rat cerebellum and cortex

Because many 5-HT functions are brain region–dependent, we expected to find a different distribution of its receptors in analogous samples from cerebellum and cortex. This was indeed the case in all samples.

Totally tissue-specific expression was found only for the subtype 5-HT_{2C}, which was proved in cerebellum from newborn and adult rats. This result contradicts some other studies. Pompeiano *et al. (26)* and Wright *et*

al. (27) found the expression of a lesser amount of 5-HT_{2C} mRNA in cerebral cortex, whereas they did not prove its expression in cerebellum. The 5-HT_{2C} receptor protein was found throughout the CNS postsynaptically to serotonergic projections *(28–30)*. The reason for these discrepancies remains unexplained; however, it could be due to different methodological approaches.

The 5-HT_{2C} subtype has attracted attention in recent years on account of studies dealing with the new therapeutic class of antidepressants called MASSA (Melatonin Agonist and Selective Serotonin Antagonist), whose leading compound is agomelatine and binds to the melatoninergic MT_1 and MT_2 receptors and to the serotoninergic 5-HT_{2C} receptors *(31)*.

Some of the receptors studied showed partial tissue specificity; for example, subtype 5-HT_{1A} was predominantly expressed in cerebellum and subtype 5-HT_6 predominantly in cerebral cortex. When interpreting results of 5-HT receptor expression studies, we must be aware of the fact that that the presence of certain receptor mRNA in a particular brain region does not automatically mean that this receptor protein is also present in this region. Vice versa, the lack of receptor information in the form of mRNA does not inevitably mean the absence of its receptor protein in the same brain part. The reason for these deviations is the functional and morphological properties of neurons, which are composed of a cell body and axons. Thus all mRNAs that come from the cell nucleus are exclusively found in cell bodies, but corresponding receptor proteins could be anchored in the membrane of the axons, which can be very long and even extend to other brain regions. Consequently, for totally precise and relevant evaluation of 5-HT receptor-tissue specificity, it would be necessary to compare the results of mRNA distribution with the receptor protein localizations.

Differences in the 5-HT receptor expression pattern between tissue homogenates and astrocyte cell cultures

It was found that tissue samples from cerebral cortex express higher number of 5-HT receptors than the corresponding astrocyte cultures. This finding was expected because the tissue contains not only astrocytes, but also other glial cells (oligodendrocytes, microglial cells), endothelial cells, progenitor cells, and of course neurons. Immunohistochemically, we proved that the astrocyte cultures are mainly composed of astrocytes type 1 (95 to 99%), the other cells in the minority being the progenitor and microglial cells. Because predominantly one cell type is present and growth space is limited, after some time cells in the culture fall into a quiescent phase, in which the expression of many proteins is reduced or even stopped. In addition, in the astrocyte cultures there are no endogenous neurotransmitters and neurohumoral factors that could affect the 5-HT receptor expression.

Of great importance is also the choice of cell-culturing reagents, especially the fetal bovine serum, because these could contain traces of mammalian proteins, neurotransmitters, and hormones that could influence receptor expression. In addition, the length of culturing is also an important parameter and can affect the receptor down- or up-regulation. For these reasons, astrocyte cultures often do not reflect the situation of *in vivo* conditions.

However, in samples from cerebellum, the number of expressed 5-HT receptor subtypes was equal in tissues and in the corresponding astrocyte cultures, although the expression patterns were not the same. Perhaps this is due to the presence of a larger number of different cell types in cortex than in cerebellum. It could also be possible that cortical astrocytes are more dependent on stimuli from neighboring cells and, when these are absent, they fall into a quiescent phase earlier. Because there are no studies in the literature to compare and interpret these results, we are preparing to perform a quantitative analysis of 5-HT receptor expression, which will help us find the answers to these questions.

The evaluation of expression of individual 5-HT receptors yielded some noteworthy conclusions. The subtypes 5-HT_{1F}, 5-HT_{2C}, and 5-HT_{5B} were predominantly expressed in tissue samples and were only occasionally found in astrocyte cultures. We could conclude that these subtypes are prone to down-regulation during culturing, perhaps due to the reasons mentioned above. It is also interesting that the subtypes 5-HT_{2B} in 5-HT_7 were predominantly expressed in astrocyte cultures, which could point to the conclusion that these two are more prone to up-regulation in culturing cells. The cause of this phenomena remains to be established.

Reports on 5-HT receptor expression in astrocytes are scant and usually do not include comparison of the results to tissue samples. Only Cohen *et al. (32)* described and compared the expression pattern of 5-HT receptor subtypes in human microvascular cells and astrocytes. In addition, Hirst *et al. (11)* determined the presence of 5-HT receptor mRNAs in astrocyte cultures prepared from neonatal Sprague-Dawley rat hypothalamic/thalamic area, and they found all but 5-HT_4 and 5-HT_{5A} receptor mRNA. In another study, they demonstrated the presence of 5-HT_6 and 5-HT_7 receptor mRNA in astrocytes from neonatal cerebral cortex and cerebellum *(33)*, which is largely consistent with our results.

We can summarize that the expression of 5-HT receptors is a process dependent on age, brain region, and probably also rat strain and cell type. The expression of 5-HT receptor subtypes in astrocyte cultures does not match the situation in corresponding brain areas. This finding continues to raise suspicion about the suitability of astrocyte cultures as a bare parallel model for their brain-region counterparts.

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