Cytoarchitecture of the mouse median raphe region

PhD thesis

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1. Introduction

The median raphe region (MRR) that is located to the brainstem contains a significant amount of serotonergic cells. The ascending pathway arising in the MRR innervates many forebrain areas, taking part in the regulation of several different cognitive functions. Besides serotonergic cells glutamatergic and GABAergic cells are also present in the MRR. However, the exact numbers and ratios of the different cell types in the MRR was unknown.

Our workgroup described previously that MRR cells can effectively and precisely excite GABAergic interneurons in the hippocampus by glutamatergic neurotransmission, having a strong effect on the hippocampal network activity. However, the the presence of this glutamatergic neurotransmission in other forebrain areas was never examined before. It is also unknown if MRR cells can innervate different forebrain areas simultaneously being able to regulate them in a synchronous manner.

In my PhD thesis I examined the cellular architecture and transmitter phenotypes of the MRR using immunofluorescent techniques and design-based stereological methods. I also examined the glutamatergic nature of the ascending pathway from the MRR using anterograde and retrograde tracing combined with correlated light- and electron microscopy.

1.1 Cell types of the MRR

Based on the classical description of Dahlström and Fuxe, the MRR consists of the B5 and B8 serotonergic cell group of the brainstem. These serotonergic cells are located to the midline of the brainstem, intermingled with glutamatergic cells expressing the type 3 vesicular glutamate transporter (vGluT3). Certain neurons express both serotonin and vGluT3, having a dual serotonergic and glutamatergic phenotype. Besides these neurons, MRR contains GABAergic and other neuropeptidergic cells expressing somatostatin or galanin in their somata.

1.2 The role of the ePet/PET-1 transcription factor system

The phenotype of the serotonergic cells in the MRR is defined by plenty of transcription factors, of which the PET-1 has an essential role. PET-1 and its homologues were described to be present in several mammalian species including humans. In mice, PET-1 expression is started at embryonic day 9.5 in the serotonergic nuclei of the brainstem, followed by the expression of different proteins playing an essential role in the serotonergic neurotransmission, like the vesicular monoamine transporter type 2, the tryptophan-hydroxylase or the serotonin plasmamembrane transporter. The PET-1 enhancer region ePet was recently described in the mouse, where it plays an essential role in the sustainment of the PET-1 activity and the serotonergic phenotype.

The number of the serotonergic cells is seriously decreased in PET-1 knockout mice, together with an impairment of the effectiveness of the serotonergic neurotransmission. However, some functional serotonergic neurons are still present in the brainstem of PET-1 knockout mice. Furthermore, the presence of PET-1 was also described in non-serotonergic cells, some of which expressed vGluT3. In spite of these controversies, we still have poor information about the exact localization of the ePet/PET-1 transcription factor system in the different cell types of the MRR.

1.3 The properties of ascending pathway from the MRR

MRR innervates several forebrain areas, where it has very specific target cells. In the hippocampus, MRR fibers innervate GABAergic interneurons located to the border of stratum radiatum and lacunosum moleculare, expressing calbindin or cholecystokinin. In the neocortex, the targets of MRR fibers are mainly $5HT_3$-type serotonin receptor expressing GABAergic cells, located to the superficial layers. In the medial septum, MRR fibers target GABAergic cells expressing parvalbumin or calbindin.

MRR fibers usually target the perisomatic region of their target neurons, establishing up to 20 boutons on a cell that suggest a very

effective modulatory mechanism. However, we did not know if single MRR cells are capable of innervating their targets located to different forebrain areas simultaneously, thus exerting a synchronous effect in these forebrain areas.

Our workgroup described before that MRR fibers can exert a very strong excitatory effect on their target cells in the hippocampus by glutamatergic neurotransmission that is mediated by AMPA-type glutamate receptors. However, the presence of the NMDA-type glutamate receptors that have a very important role in synaptic plasticity mechanisms were not examined in the synapses established by MRR in the hippocampus. Neither do we know if MRR fibers are capable of glutamatergic neurotransmission in other forebrain areas.

2. Scientific objectives

I. My first goal was to define the exact numbers and ratios of the different cell types located to the MRR. I also examined the overlap between the different cell populations. Finally, I examined that how specific is the ePet/PET-1 transcription factor system on the MRR serotonergic cells.

II. My second task was to examine the abundance of the nonserotonergic, glutamatergic component of the ascending MRR pathway.

III. I examined the presence of postsynaptic NMDA-receptors in the synapses established by MRR in three different forebrain areas (hippocampus, medial septum and medial prefrontal cortex).

IV. Finally I examined if MRR cells can innervate different forebrain areas simultaneously.

3. Materials and methods

3.1 Animals and stereotaxic surgeries

For stereologic experiments we used 3 pieces of vGAT-IRES-Cre::Gt(ROSA26)Sor_CAG/ZsGreen1, 1 piece of vGAT-IRES-Cre::Gt(ROSA26)Sor_CAG/tdTomato, 3 pieces of ePet-IRES-Cre::Gt(ROSA26)Sor_CAG/ZsGreen1 and 1 piece of C57Bl/6 wild type mice. For tracing experiments we used 15 pieces of C57Bl/6 wild type and 1 vGluT3 knockout mice. For tracing experiments we deeply anaesthetized mice with a mixture of ketamine and xylazine dissolved in saline. This was followed by stereotaxic injection of the following tracers: for anterograde tracing 5-10 nl of 10 kDa biotinylated dextrane amine (Molecular Probes) into the MRR; for retrograde tracing 14-18 nl of FluoroGold (Fluorochrome Inc.) into the medial prefrontal cortex (mPFC) or 18-23 nl of choleratoxin B subunit (CTB; List Biologicals) into the medial septum (MS) or hippocampus (HIPP). After surgeries, animals received the anti-inflammatory drug meloxicam (Boehringer-Ingelheim), and survived in separate cages for 8-14 days before perfusions.

3.2 Fluorescent immunohistochemostry

Mice were perfused through the heart by freshly depolymerized 4% paraformaldehyde for 40 minutes, followed by the removal of the brains from the skull and preparing of 50-60 μm thick coronal slices with a vibratome. After subsequent washes and preincubation for antigen retrieval and removal of aspecific background, sections were incubated in solutions containing the following primary antibodies: rabbit-anti-FluoroGold (1:10000-1:30000, Millipore), mouse-anti-CTB (1:500, Abcam), goat-anti-CTB (1:20000, List Biologicals), mouse-anti-NeuN (1:500, Chemicon), rabbit-anti-serotonin (1:10000, ImmunoStar), rat-anti-serotonin (1:500, Millipore), mouse-anti-tryptophan-hydroxylase (1:3000, Sigma-Aldrich), rabbit-anti-vGluT3 (1:500, Synaptic Systems) and guinea pig-anti-vGluT3 (1:1000, Millipore). 72 hours later the sections were incubated in the solution of the following secondary antibodies: Alexa488-conjugated donkey-anti-mouse (1:500, Life

Technologies) or donkey-anti-rabbit (1:500-1:1000, Life Technologies) or goat-anti-guinea pig (1:500, Molecular Probes); DyLight549-conjugated donkey-anti-mouse (1:500, Jackson ImmunoResearch); Alexa594-conjugated donkey-anti-mouse (1:500, Life Technologies) or donkey-anti-goat (1:500, Life Technologies); Cy3-conjugated donkey-anti-rabbit (1:500, Jackson ImmunoResearch); Alexa647-conjugated chicken-anti-rat (1:500, Life Technologies) or donkey-anti-rabbit (1:500, Jackson ImmunoResearch); or biotinylated goat-anti-guinea pig (1:200, Vector Laboratories), that was converted into the fluorescent channel by using DyLight405-conjugated streptavidin (1:500, Jackson ImmunoResearch). We paid attention to test the specificity of our primary antibodies in knock out animals, and possible cross-reactions between the different primary and secondary antibodies was also exhaustively tested. For stereological experiments, nuclei were visualized using DAPI counterstaining (1:10000, Sigma-Aldrich). Finally, sections were embedded into Aquamount (BDH Chemicals LTD) and they were evaluated using a Zeiss Axioplan 2 epifluorescent microscope or an Olympus Optical FluoView 300 or a Nikon A1R laser-scanning confocal microscope system.

3.3 Stereological measurements

For stereological measurements, sections containing the MRR were collected into 8 vials with systematic random sampling. We performed two kinds of fluorescent immunoreactions: on every odd numbered vial we labeled serotonin- or vGluT3-positive cells in different channels; on every even numbered vial, we labeled serotonin- or vGluT3-positive cells in the same channel, and labeled other neurons in a separate channel. MRR was reconstructed in z-stacks with 1 μm focal plane distance using the confocal microscope, and we measured the numbers of the different cell types using the optical fractionator method. In total, we counted about 13% of the MRR cell population (n=12300 cells in 8 mice). For the measurements we used the software StereoInvestigator (MicroBrightField Bioscience); the neurochemical properties of the counted cells were defined parallelly using the NIS-Elements Documentation software.

3.4 Preembedding immunohistochemistry and electron microscopy

Sections were preincubated for antigen-retrieval and blocking of aspecific background labeling. Pepsin digestion was performed for the retrieval of the GluN2A subunit of the NMDA-receptors. These were followed by incubation in the solution of the following primary antibodies: rabbit-anti-GluN2A subunit (1:150-1:250, gift of Masahiko Watanabe, Dept. of Anatomy, Hokkaido University, Japan), rabbit-anti-Neuroligin 2 (1:600, Synaptic Systems), and guinea pig-anti-vGluT3 (1:4000, Millipore). 72 hours later this was followed by incubation in the solutions of the following secondary antibodies: biotinylated goat-anti-guinea pig (1:200, Vector Laboratories) and 1.4nm gold-conjugated goat-anti-rabbit (1:100-1:300, Nanoprobes). The vGluT3- or BDA-labeling was developed by immunoperoxidase reaction; for this, the sections were incubated in ABC Elite (1:300, Vector Laboratories), and for the development DAB (Sigma-Aldrich) was used as a chromogen. GluN2A- and Neuroligin 2-labeling was further developed by silver-intensification (SE-EM kit, Aurion). Following this, sections were treated with 0.5% osmium-tetroxide, and they were dehydrated with ascending alcohol series and acetonitrile. During dehydration, sections were treated with 1% uranyl-acetate for contrast enhancement. Finally, sections were embedded into an epoxy resin (Durcupan, ACM, Fluka). Samples from the embedded sections were reembedded onto epoxy resin blocks, and we cut 70-100 nm thick ultrathin sections from them using a
Leica EM UC6 ultramicrotome. Ultrathin sections were evaluated using a Hitachi H-7100 electron microscope and a Veleta CCD camera (Olympus). The examined synapses were always fully reconstructed, and stereological rules were observed during the evaluation procedure.

4. Results

4.1 Description of the cellular architecture of the MRR

Using unbiased, design-based stereological methods, we managed to describe the exact numbers and ratios of the different cell types present in the mouse MRR. We found that the mouse MRR contains about 47500 neurons. About 60% of these cells are GABAergic (approx. 29000 pcs), not overlapping with any other cell types in the MRR. The most thoroughly studied serotonin- and/or vGluT3-positive cells comprise only about 13% of the MRR neuron population (about 6000 cells). About one-sixth (approx. 1000 pcs) of these cells contains only serotonin, about one-fourth (approx. 1700 pcs) contains both serotonin and vGluT3, and the rest of them (approx. 3300 pcs) contains only vGluT3. About 25% of the MRR cells (approx. 11000 pcs) belong to a so far unidentified population that does not express GABA, serotonin or vGluT3; the exact neurochemical identity of these cells is still unknown.

Besides the neurochemical description of MRR cells we found that the ePet/PET-1 transcription factor system is not specific to the serotonergic cells, in contrast to the data found in the literature. According to our measurements, only about 70% of the serotonin-expressing cells contain ePet, while about the half of the cells expressing only vGluT3 also expressed ePet. GABAergiccells and those neurons that belong to the unidentified population did not express ePet in their somata.

4.2 Innervation pattern of MRR projection cells in the forebrain

We labeled MRR cells projecting to the HIPP, MS or mPFC using FluoroGold and CTB as retrograde tracers, and we examined their serotonin- and vGluT3-positivity using immunofluorescent stainings. According to our measurements, at least about 80% of the MRR cells projecting to the HIPP, 60% of the MRR cells projecting to the MS and 70% of the MRR cells projecting to the mPFC expressed vGluT3 in their somata, therefore these cells were considered to be glutamatergic. Only serotonin-positive cells comprised only about 10% of the ascending MRR pathway. These results correlated well with the ratios of the different cell types in the MRR, and show that the ascending MRR pathway

communicates primarily through glutamatergic transmission with its targets.

4.3 MRR cells project to more forebrain areas simultaneously

Using double retrograde tracing we examined if single MRR cells can innervate more forebrain areas simultaneously. According to our measurements, at least about 10% of the MRR cells innervate mPFC and HIPP, and at least about 15% of them innervates mPFC and MS simultaneously. Using fluorescent immunohistochemistry, we examined the neurochemical identity of these double-projecting cells: according to our measurements at least about 80% of them is vGluT3-positive, while only about 10% of them contains only serotonin. Therefore these cells might regulate network activity in different forebrain areas in a sychronous manner very effectively by fast glutamatergic neurotransmission.

4.3 MRR synapses established in the forebrain contain NMDA-receptors

Using anterograde tracing and correlated light- and electron microscopy we examined if forebrain MRR synapses contain the GluN2A subunit of the NMDA-receptors. At least about 30% of MRR synapses established in the HIPP, at least about 50% of the MRR synapses established in the MS, and at least about 60% of the MRR synapses established in the mPFC contained GluN2A-labeling in the postsynaptic side. The density of the immunogold labeling in MRR synapses and local asymmetric synapses was in the same range in every examined forebrain area. This suggests that MRR can very effectively activate its target cells in the forebrain by glutamatergic neurotransmission activating NMDA-receptors.

5. Conclusions

This is the first description of the cellular architecture of the MRR using statistically unbiased methods. The examination of the neurochemical phenotype of MRR neurons showed the surprising results that the majority, about 60% of these cells are GABAergic, only about 15% of them belongs to the most thoroughly studied group of serotonin- and/or vGluT3-positive cells, and about 25% of them belongs to a so far unidentified population, the neurochemical identity of which is unknown. We also showed that the ePet/PET-1 system considered to be specific to serotonergic cells is also present in about half of the cells expressing only vGluT3. These results suggest that the reconsideration of the data available in the literature about the ePet/PET-1 system is necessary.

Available data suggest that the serotonin and/or vGluT3-positive neurons build up the ascending MRR pathway. Our results show that the majority of this pathway is established by vGluT3-positive cells, comprising even about 80% of the projection to the examined forebrain areas. Our double retrograde tracing experiments showed that single MRR cells are capable to innervate more forebrain areas simultaneously, and the majority of the double projecting cells also express vGluT3. These results suggest that MRR can very effectively regulate network activity in these brain areas in a synchronous manner, by the glutamatergic activation of its GABAergic target neurons.

The previous results of our workgroup showed that MRR cells can activate hippocampal GABAergic neurons located to the border of stratum radiatum and lacunosum-moleculare by a fast and precise glutamatergic neurotransmission, having a strong effect on the network activity of the hippocampal pyramidal cells.

In this work we showed the presence of AMPA-receptors on the postsynaptic side of raphe-hippocampal synapses, and now we showed that NMDA-receptors are also present in these synapses. Furthermore, the presence of NMDA-receptors was also shown in the synapses established by MRR in the other examined forebrain areas, suggesting the presence of functional glutamatergic transmission. The NMDA-receptors in these synapses might play a role in synaptic plasticity mechanisms and they might be an important target of local regulatory mechanisms.

In conclusion, MRR is in an ideal position to exert a fast, synchronous effect through glutamatergic transmission in the forebrain that is supported by serotonergic modulation.

6. Publication list of the author

Original publications related to the PhD thesis:

Sos KE, Mayer MI, Cserep C, Takacs FS, **Szonyi A,** Freund TF, Nyiri G
Cellular architecture and transmitter phenotypes of neurons of the mouse median raphe region
BRAIN STRUCTURE & FUNCTION 222: (1) pp. 287-299. (2017)
IF: 4,698*

Szonyi A, Mayer MI, Cserep C, Takacs VT, Watanabe M, Freund TF, Nyiri G
The ascending median raphe projections are mainly glutamatergic in the mouse forebrain
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IF: 4,698

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Takacs VT, **Szonyi A,** Freund TF, Nyiri G, Gulyas AI
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