Visualisation and Characterisation of Functional 5-HT Receptors in the Human Dorsal Raphe Nucleus by [35S]GTPγS Autoradiography

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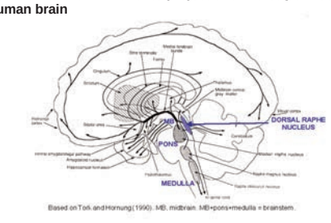
Introduction

- The dorsal raphe nucleus (DRN) comprises a collection of neurones lying along the mid-line of the brainstem, extending from the posterior midbrain caudally to the anterior pons (Fig. 1). Most DRN neurones contain 5-HT and give rise to axons which project to the forebrain.
- 5-HT receptors in the DRN are suggested to be a site of action of some antidepressant drugs (Briley and Moret, 1992).
- 5-HT receptors belong to the superfamily of G-protein-coupled receptors (GPCRs). Agonist-stimulated activation of some GPCRs has been studied in tissue membranes by measuring [^{35}S]GTPγS binding following stimulation with agonist. In this way, it is possible to generate quantitative, pharmacological data on native and recombinant receptors.
- Agonist-stimulated [^{35}S]GTPγS binding can be applied to tissue sections, where bound [^{35}S]GTPγS is detected by autoradiography. This allows anatomical resolution of receptor function in discrete regions of the brain that would not be accessible for membrane binding studies.
- To date, there are few [^{35}S]GTPγS autoradiography studies in human brain tissue (Day et al, 1999a and b; Dupuis et al, 1999) and virtually no studies producing pharmacological data in animal or human tissue (Day et al, 1999a).
- We have used [^{35}S]GTPγS autoradiography to visualize and characterize the activation of 5-HT receptors in the human DRN. The study was performed in two stages. In Stage 1, 5-HT-stimulated [^{35}S]GTPγS binding was mapped through the suspected rostrocaudal extent of the DRN. In Stage 2, 5-HT receptors in the DRN were characterized by performing 5-HT concentration-effect curves in the absence and presence of the specific 5-HT$_{1A}$ receptor antagonist, WAY100635.

Figure 1. Location of the DRN and its projections in a sagittal section of the human brain



Based on Tork and Hornung (1990). MB: midbrain. MP=pons+medulla = brainstem.

Tissues

Human brain tissue was obtained at autopsy from four donors with no prior history of neurological disease. Donor details are shown in Table 1. Whole coronal blocks of midbrain and pons were dissected from the brainstem and either frozen on a brass plate cooled on dry ice or in isopentane cooled in liquid nitrogen. Coronal sections (15µm) of midbrain and pons were cut through the suspected rostrocaudal extent of the DRN. Sections were stored at –80ºC until use.

Table 1: Donor information

Donor	Sex	Age	*Fz delay (h)	Cause of death	Significant clinical diagnosis
1	M	77	28	Multiple myelomas of the spine	Multiple myelomas of the spine
2	M	80	11	Old age	Mild emphysema, acute cystitis
3	F	86	28	Hypotensive cardiomyopathy	Long-standing motor peripheral neuropathy
4	F	49	38	Breast cancer: liver metastases	Breast cancer:liver metastases

Methods

[^{35}S]GTPγS autoradiography

Experimental protocol

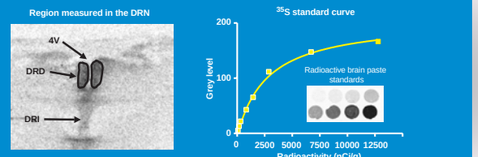
Assay buffer: 50mM Tris-HCl (pH7.4), 3mM $MgCl_2$, 0.2mM EGTA, 100mM NaCl, 0.3-0.6mM GDP, 1mM DTT.

1) Fresh frozen sections were pre-incubated in assay buffer ± WAY100635 (30nM) for 30min at 25°C.
2) Sections were then incubated in assay buffer with 0.1nM [35S]GTPgS, 0.5mM ascorbic acid and 10µM pargyline, ± 5-HT (0-100µM), ± WAY100635 (30nM), for 2h at 25∞C.
3) Sections were washed in 50mM Tris-HCl (pH7.4), 2x3min at 4oC, followed by dipping in H2O (4°C).
4) Sections were then air-dried and apposed to Kodak BMR film, overnight, alongside radioactive brain paste standards.
5) The film was developed in Photosol RG developer.
6) Autoradiographic images were scanned to PC using a UMAX PowerLook III scanner.

Quantification

1) Grey level measurements of [^{35}S]GTPγS binding to the DRN were made in the dorsal part of the DRN (DRD) immediately adjacent to the 4th ventricle by drawing around the region of interest (see Fig. 2; NIH Image software used).
2) Grey level measurements of the DRD were converted to nCi/g using the ^{35}S standard curve (Fig. 2). All grey levels from DRD measurements fell within the lower portion of the curve (highest grey level was 105).
3) The concentration-effect data were analysed using GraphPad Prism software.

Figure 2. Quantification of 5-HT-stimulated [^{35}S]GTPγS binding in the DRD



Results

Stage 1: Mapping of 5-HT-stimulated [^{35}S]GTPγS binding in the DRN

In Stage 1, 5-HT-stimulated [^{35}S]GTPγS binding was mapped through the suspected rostrocaudal extent of the DRN. Three adjacent 15µm sections were sampled every fifty sections from each donor. Two sections were subjected to [^{35}S]GTPγS autoradiography in the absence (basal) and presence of 10µM 5-HT. The third adjacent section was stained for acetylcholinesterase (AChE) using standard methodology.

Figures 4 to 7 show 5-HT-stimulated [^{35}S]GTPγS binding at two levels of the DRN, trochlear nucleus and 4th ventricle, identification of which was based on anatomical features shown by AChE staining. At these levels, the DRN is composed of several subnuclei, as shown in Fig. 3. In all four donors, 5-HT (10µM) caused an increase in [^{35}S]GTPγS binding over basal levels in the region of the DRN. However, the pattern of stimulation in the DRN subnuclei was slightly different for each donor.

Figure 3. Partial hemi-coronal sections of the brainstem showing subnuclei of the DRN

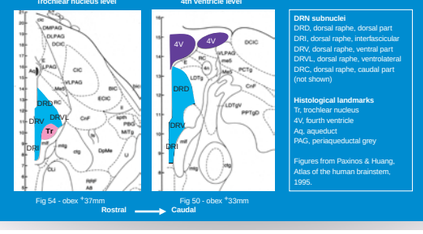
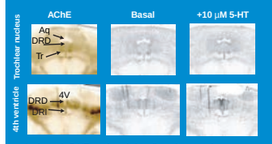
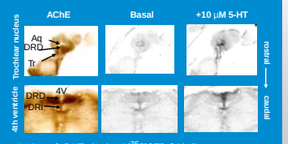


Figure 4. 5-HT-stimulated [^{35}S]GTPγS binding in donor 1



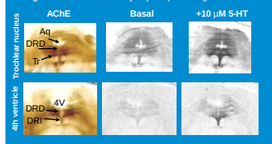
In donor 1, 5-HT produced virtually no increase in [^{35}S]GTPγS binding at the level of the trochlear nucleus, while marked stimulation was observed at the level of the 4th ventricle in the DRD, immediately adjacent to the 4th ventricle.

Figure 5. 5-HT-stimulated [^{35}S]GTPγS binding in donor 2



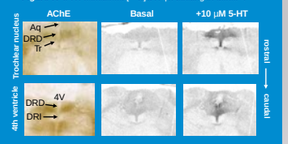
In donor 2, 5-HT-stimulated [^{35}S]GTPγS binding was observed at the level of the trochlear nucleus, just ventral to the aqueduct. At the level of the 4th ventricle, stimulation was observed in the DRD (adjacent to the ventricle) and DRI (DRD > DRI).

Figure 6. 5-HT-stimulated [^{35}S]GTPγS binding in donor 3



In donor 3, 5-HT-stimulated [^{35}S]GTPγS binding at the level of the trochlear nucleus was more widespread than seen for donors 1 and 2. Stimulation was observed in the DRD, DRI and the medial and lateral DRV (the latter not apparent for donors 1 and 2). This pattern of stimulation closely mirrors that observed for [^{3}H]8-OH-DPAT (5-HT^{1A} receptor agonist) binding in the human DRN (Stockmeier et al, 1996). At the level of the 4th ventricle, stimulation was observed in the DRD, DRV and DRI (DRI > DRD).

Figure 7. 5-HT-stimulated [^{35}S]GTPγS binding in donor 4



In donor 4, stimulation at the level of the trochlear nucleus was fairly widespread (as seen for donor 3) with an increase in [^{35}S]GTPγS binding in the DRD, DRV and DRI. At the level of the 4th ventricle, stimulation was apparent in these same regions.

Stage 2: 5-HT concentration-effect curves and antagonism with WAY100635

In Stage 2, 5-HT concentration-effect curves (0 – 100µM) were performed on a series of DRN sections containing the DRD (at the level of the 4th ventricle), in the absence and presence of 30nM WAY100635. Duplicate sections were used for each incubation condition. Grey level measurements of [^{35}S]GTPγS binding were made in the DRD, as shown in Fig. 2.

In this experiment, no 5-HT stimulation of [^{35}S]GTPγS binding was observed for donor 1 (results not shown), despite stimulation being seen in Stage 1. The reason for this lack of stimulation is not clear, but may be due to a combination of factors, including section storage time and factors specific to the donor. Antagonism of 5-HT-stimulated [^{35}S]GTPγS binding in the DRN by WAY100635 is demonstrated autoradiographically in Figure 8 (donor 4 only). Concentration-effect curves for donors 2, 3 and 4 are shown in Figure 9.

Figure. 8 Antagonism of 5-HT-stimulated [^{35}S]GTPγS binding by WAY100635 (donor 4): autoradiographic images

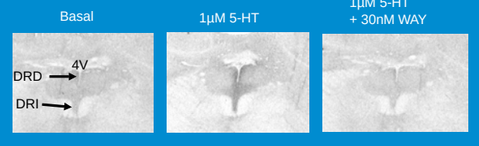
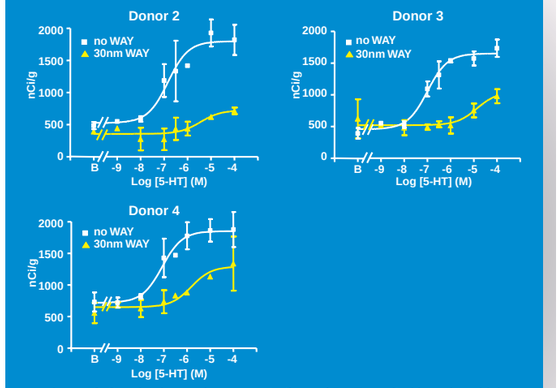


Table 2: Analysis of data from 5-HT concentration-effect curves (data from Fig. 9)

	5-HT		5-HT + 30nM WAY100635	
Donor	**pEC50**	**% of basal at 100µM 5-HT**	**pEC50**	**% of basal at 100µM 5-HT**
2	6.8	331	5.5	161
3	7.0	313	4.9	188
4	7.1	260	5.6	196
mean	**7.0 ± 0.1**	**301 ± 21**	**5.3 ± 0.2**	**182 ± 11**

Figure. 9 Antagonism of 5-HT-stimulated [^{35}S]GTPγS binding in the DRD by WAY100635: concentration-effect curves



In donors 2, 3 and 4, increasing concentrations of 5-HT resulted in a sigmoidal increase in [^{35}S]GTPγS binding in the DRD. In the presence of WAY100635 (WAY), the 5-HT curves were shifted to the right. However, the antagonism produced by WAY100635 did not appear to be surmountable. The reason for this is not clear. The curves were analysed to produce pEC_{50} values and the % stimulation at 100µM 5-HT (Table 2). These data produced an apparent pK_b for WAY100635 of 9.0 ± 0.2.

SUMMARY AND DISCUSSION

- 5-HT (10µM) caused an increase in [^{35}S]GTPγS binding over basal levels in the DRN of four donors, although the spatial localization of stimulation was slightly different for each donor.
- The pattern of 5-HT-stimulated [^{35}S]GTPγS binding was relatively discrete for donors 1 and 2 (male), but more widespread for donors 3 and 4 (female). The reason for this is not clear, although it may relate to gender differences or other differences in donor genotype/phenotype.
- For donors 3 and 4, the pattern of stimulation at the level of the trochlear nucleus closely mirrors that observed for [^{3}H]8-OH-DPAT (5-HT$_{1A}$ receptor agonist) binding in the human DRN (Stockmeier et al, 1996).
- 5-HT stimulated [^{35}S]GTPγS binding in the DRD to a maximum of 260-331% of basal binding, with a mean pEC_{50} of 7.0.
- The selective 5-HT$_{1A}$ antagonist, WAY100635, antagonised 5-HT-stimulated [^{35}S]GTPγS binding in the DRD, in a non-surmountable fashion. The reason for this is not clear, but is the subject of further investigation.
- WAY100635 antagonized 5-HT-stimulated [^{35}S]GTPγS binding in the DRD with an apparent pK_b of 9.0 which is consistent with the reported affinity of this ligand for human recombinant 5-HT$_{1A}$ receptors (Fletcher et al, 1996).
- This study demonstrates the feasibility of using [^{35}S]GTPγS autoradiography to obtain quantitative, pharmacological data for GPCRs in small regions of human brain and suggests that 5-HT receptor-stimulation of [^{35}S]GTPγS binding in the DRN is mediated almost exclusively by 5-HT$_{1A}$ receptors.

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