

Research Article

A patterned architecture of monoaminergic afferents in the cerebellar cortex: noradrenergic and serotonergic fibre distributions within lobules and parasagittal zones

Michael Longley, John Ballard, Maria Andres-Alonso, Rebecca Constance Varatharajah, Hadleigh Cuthbert, Christopher H. Yeo

PII: S0306-4522(20)30565-0

DOI: <https://doi.org/10.1016/j.neuroscience.2020.09.001>

Reference: NSC 19857

To appear in: *Neuroscience*

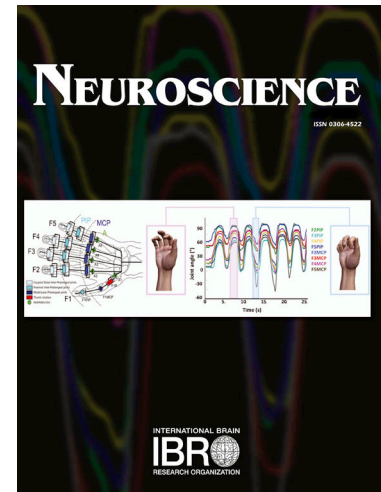
Received Date: 27 April 2020

Revised Date: 28 August 2020

Accepted Date: 1 September 2020

Please cite this article as: M. Longley, J. Ballard, M. Andres-Alonso, R. Constance Varatharajah, H. Cuthbert, C.H. Yeo, A patterned architecture of monoaminergic afferents in the cerebellar cortex: noradrenergic and serotonergic fibre distributions within lobules and parasagittal zones, *Neuroscience* (2020), doi: <https://doi.org/10.1016/j.neuroscience.2020.09.001>

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



Title: A patterned architecture of monoaminergic afferents in the cerebellar cortex: noradrenergic and serotonergic fibre distributions within lobules and parasagittal zones.

Authors: Michael Longley¹, John Ballard, Maria Andres-Alonso², Rebecca Constance Varatharajah, Hadleigh Cuthbert and Christopher H. Yeo

Affiliations: Dept. Neuroscience, Physiology and Pharmacology, University College London, London WC1E 6BT, U.K.

Email: ML – michael.Longley@rhul.ac.uk; JB - jxb283@doctors.org.uk; MRAA - maria.andres-alonso@zmnh.uni-hamburg.de; RV - rebecca.v@doctors.org.uk; HC - hadleigh.cuthbert.11@alumni.ucl.ac.uk; CHY – c.yeo@ucl.ac.uk

Corresponding author: Prof. Christopher Yeo
Dept. Neuroscience, Physiology and Pharmacology,
University College London,
London WC1E 6BT,
U.K.
Email: c.yeo@ucl.ac.uk

Declarations of interest: none

¹ Department of Psychology, Royal Holloway University of London, Egham, United Kingdom

² Center for Molecular Neurobiology, ZMNH, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

ABSTRACT

The geometry of the glutamatergic mossy-parallel fibre and climbing fibre inputs to cerebellar cortical Purkinje cells has powerfully influenced thinking about cerebellar functions. The compartmentation of the cerebellum into parasagittal zones, identifiable in olivo-cortico-nuclear projections, and the trajectories of the parallel fibres, transverse to these zones and following the long axes of the cortical folia, are particularly important. Two monoaminergic afferent systems, the serotonergic and noradrenergic, are major inputs to the cerebellar cortex but their architecture and relationship with the cortical geometry are poorly understood. Immunohistochemistry for the serotonin transporter (SERT) and for the noradrenaline transporter (NET) revealed strong anisotropy of these afferent fibres in the molecular layer of rat cerebellar cortex. Individual serotonergic fibres travel predominantly medial-lateral, along the long axes of the cortical folia, similar to parallel fibres and Zebrin II immunohistochemistry revealed that they can influence multiple zones. In contrast, individual noradrenergic fibres run predominantly parasagittally with rostral-caudal extents significantly longer than their medial-lateral deviations. Their local area of influence has similarities in form and size to those of identified microzones. Within the molecular layer, the orthogonal trajectories of these two afferent systems suggest different information processing. An individual serotonergic fibre must influence all zones and microzones within its medial-lateral trajectory. In contrast, noradrenergic fibres can influence smaller cortical territories, potentially as limited as a microzone. Evidence is emerging that these monoaminergic systems may not supply a global signal to all of their targets and their potential for cerebellar cortical functions is discussed.

KEYWORDS:

Cerebellar cortex

Monoamines

5-HT

Noradrenaline

Cerebellar zones

INTRODUCTION

There are several types of neuromodulatory afferents to the cerebellum including four that express the monoamines: 5-HT, dopamine, histamine and noradrenaline. By afferent number, the two most significant are serotonergic (5-HT) and noradrenergic (NA) (Ito 1984; Kerr and Bishop 1991; Schweighofer et al. 2004). Serotonergic and noradrenergic fibres distribute widely across the cerebellar cortex, through each of its three layers, and through the cerebellar nuclei (see for example: Bishop and Ho 1985; Bloom et al. 1971; Chan-Palay 1975; Di Mauro et al. 2003; Hökfelt and Fuxe 1969; Takeuchi et al. 1982). Both types are “beaded” afferents with varicosities along their lengths. It is generally assumed that propagated activity will lead to release from all varicosities along the entire afferent axon. These afferents can exert a wide influence on the cerebellum because the main cerebellar cortical neuronal types all express either 5-HT receptors (reviewed by Geurts et al. 2002), or NA receptors (Hirono et al. 2008; Papay et al. 2006; Papay et al. 2004; Paschalis et al. 2009). These serotonergic and noradrenergic afferents are significant inputs to cerebellar neurons but the contribution of these potentially information-rich monoaminergic signalling systems to cerebellar functions is not well-understood.

Here, we have examined the patterning of noradrenergic and serotonergic afferent systems and how they relate to the precise and regularly-repeating neuronal arrays of the cerebellar cortex (Eccles et al. 1967). This repeating structure suggests that a similar computation may be implemented in each cortical array (Albus 1971; Gilbert 1974; Ito 1982; Marr 1969; Yeo and Hesslow 1998) such that functional differences between regions of the cerebellar cortex mainly depend upon their different afferent and efferent connections (Porrill et al. 2013). The glutamatergic mossy fibre (MF) and climbing fibre (CF) afferents to these arrays have been characterised in great detail and provide evidence of functionally-specific, anatomically-mapped territories within which the monoaminergic systems distribute. MF and CF afferent systems provide indirect and direct input to Purkinje cell dendrites, respectively (Fig. 1A). Climbing fibres from different sub-nuclei of the inferior olive terminate in discrete, rostral-caudal *zones* (Fig. 1B)³. The zones are further subdivided into microzones that are innervated by subsets of CFs with similar response properties that relate to the sensory inputs of their olivary source neurons (Andersson and Oscarsson 1978; Buisseret-Delmas and Angaut 1993; Ekerot and Larson 1979a; Ekerot and Larson 1979b; Garwicz and Ekerot 1994; Hesslow 1994a; Hesslow 1994b; Oscarsson 1979; Sugihara and Shinoda 2004; Voogd and Bigare 1980). Mossy fibres terminate on granule cells, the axons of which ascend to the molecular layer, bifurcate and extend several millimetres medio-laterally as parallel fibres

³ For simplicity, we here describe anatomical arrangements and orientations as they appear in the midline vermis, which was the source of the experimental material. Thus rostral-caudal is equivalent to a parasagittal plane and is orthogonal to the long-axes of the cerebellar folia. Medial-lateral is equivalent to a transverse plane and is parallel to the long axes of the cerebellar folia. The anatomical relationships we go on to describe strictly relate to the foliar axes, which diverge laterally and into the hemispheres. Hence, the terms parasagittal, transverse and longitudinal refer to the foliar long axes throughout, rather than to strict, Cartesian coordinates

(PFs) (Coutinho et al. 2004; Napper and Harvey 1988; Pichitpornchai et al. 1994). Because of their long trajectories, PFs pass through several hundred PC dendritic arbours and make synaptic contact with many (Harvey and Napper 1988). Thus, a single MF afferent can provide input to PCs across several zones and multiple microzones.

This characteristic architecture of the MF and CF glutamatergic afferent systems has inspired many leading theories of cerebellar function (Albus 1971; Dean et al. 2010; Gilbert 1974; Ito 1982; Marr 1969; Miles and Lisberger 1981). But the monoaminergic afferent systems have received less theoretical attention. Gilbert (1975) was the first to suggest a role for NA in cerebellum-dependent memory consolidation; later it was suggested that NA provides a global signal that gates plasticity mechanisms (Schweighofer et al. 2004). In both accounts, plasticity processes are initiated only in neurons that receive contingent activation of contiguous MF and CF glutamatergic inputs, so the NA signal could be global with no necessity for individual NA fibres to be restricted in their distribution. This view of NA signalling is consistent with the traditional view that NA afferents provide a diffuse and homogenous signal across much of the brain (Aston-Jones and Cohen 2005; Berridge and Waterhouse 2003; Bouret and Sara 2005; Sara and Bouret 2012).

Each of the main cerebellar neuronal types express NA and 5-HT receptors with subtypes mostly identified. There has been particular interest in exploring 5-HT influences on information processing in the cortex, through its modulatory action upon Purkinje cells and upon inhibitory interneurons, including basket and stellate cells (Dieudonné and Dumoulin 2000) and Lugaro cells (Dean et al. 2003; Dieudonné and Dumoulin 2000). In their theoretical account of cerebellar monoamine functions, Schweighofer et al. (2004) suggested that 5-HT signalling coordinates processes across a set of multiple microzones that together could provide an internal model for a single action. At present, there is less empirical evidence to support this proposal. If, as might be expected, a set of microzones needed for control of a single action were distributed across non-contiguous cerebellar cortical territories (Apps and Garwicz, 2005) it is not clear how 5-HT release from the entire length of a beaded fibre might be capable of selecting such non-contiguous regions.

The presence of cerebellar noradrenergic and serotonergic afferents has been reported widely (Beaudet and Sotelo 1981; Bishop and Ho 1985; Felten et al. 1986; Hökfelt and Fuxe 1969; Kimoto et al. 1981; Kitzman and Bishop 1994; Landis and Bloom 1975; Landis et al. 1975; Mugnaini and Dahl 1975; Verney et al. 1982; Walker et al. 1988; Yamamoto et al. 1977). Some of these accounts noted that the trajectories of 5-HT afferents are similar to those of parallel fibres (Hökfelt and Fuxe, 1969; Chan-Palay, 1975; Sur et al. 1996) and others recognised that this similarity to parallel fibres was lobule-dependent and different patterns can be seen, especially in flocculus and paraflocculus (Takeuchi et al. 1982; Bishop and Ho 1985). Noradrenergic fibres are reported to form a more diffuse plexus with no clear trajectory preferences (Hökfelt and Fuxe 1969). To date,

there has been no systematic analysis of the geometrical organisation of these serotonergic and noradrenergic afferents in relation to known zonal and microzonal architecture of the cerebellar cortex.

Antibodies raised to the serotonin reuptake transporter (anti-SERT) and noradrenaline transporter (anti-NET) are reliable markers of 5-HT and NA fibres, respectively (Nielsen et al. 2006; Qian et al. 1995; Schroeter et al. 2000; Zhang et al. 2013). Here we have used SERT and NET immunoreactivity (IR) of cerebellar vermis to reveal the full trajectories of the afferent fibres and the geometry of their distribution relative to the zonal and microzonal architecture of the cerebellar network. We reveal remarkable orthogonal anisotropies in the trajectories of these two afferent fibre types that give strong clues to functional properties of these different neuromodulatory systems.

EXPERIMENTAL PROCEDURES

Animals and preparation of materials

Seventeen adult female Sprague Dawley rats (weight: approximately 250g) were used. All procedures were approved by the local ethical review panel of University College London and were in accordance with the UK Animals (Scientific Procedures) Act 1986 and associated guidelines.

Each rat was anaesthetised with isoflurane by inhalation (4% in O₂) then killed by barbiturate overdose (200 mg/kg i.p.) of Euthatal (pentobarbitone sodium, 200 mg/ml, Merial, Essex, U.K.). Rats were perfused transcardially, first with normal saline solution (0.9 % w/v) containing heparin sodium (1000 IU/l) and then with fixative (4% w/v paraformaldehyde (PFA)/ 0.5 % w/v picric acid in 0.1 M phosphate buffer, pH 7.5). The brains were removed and post-fixed overnight in PFA solution (4% w/v paraformaldehyde in 0.1 M phosphate buffer, pH 7.5), then transferred to 20 % w/v sucrose in 4% PFA and stored for ~72 hours for cryoprotection and further fixation.

Preliminary results had indicated that serotonergic and noradrenergic afferents differed markedly in their overall trajectories. To capture these distributions, it was necessary to measure trajectories in sections cut in at least two orthogonal planes. We cut serial sections of 6 frozen cerebellums at 40 µm in the coronal plane and of 6 more frozen cerebellums at 40 µm in the parasagittal plane, using a sledge microtome (Leitz 1400; Leica, Germany). For true parasagittal sections close to the midline, fibre trajectories perpendicular to the long axis of the folium (perpendicular to parallel fibres) can be estimated with accuracy because the fibres will be

contained for most of their length within the section. The same is not true for measures of fibre trajectories along the long axis of the folium (similar to parallel fibre trajectories) made in coronal sections. Such fibres can exit the coronal sections because of additional foliar curvature in the rostral-caudal direction so thicker sections can reduce measurement error. For this reason, we also made serially-sectioned 5 frozen cerebellums at 200 μm , in the coronal plane (see below).

Immunohistochemistry

Prior to beginning conventional immunohistochemistry procedures, the 200 μm sections underwent treatment to help improve antibody penetration. They were put through three freeze-thaw cycles at $-50\text{ }^{\circ}\text{C}$ for 20 minutes and then 45 minutes at ambient temperature. They then underwent a mild and short treatment with a low concentration of proteinase K (10 $\mu\text{g}/\text{ml}$, for 15 minutes). Finally they were washed with 0.1 M Tris-buffered saline (TBS) three times for 20 minutes to remove residual proteinase K.

The blocking solution used in all subsequent procedures contained normal goat serum (2.5 % w/v), Tween 100 (0.05 % w/v), bovine serum albumin (1 %) and Triton X (0.8 % w/v for 200 μm sections or 0.2 % w/v for 40 μm sections). All antibodies were diluted using the 0.2 % w/v Triton X blocking solution to concentrations detailed in Table 1 (Supplementary Material). Washes were three x 10 minutes in 0.1 M TBS for 40 μm sections and three x 40 minutes for 200 μm sections.

40 μm sections were incubated in blocking solution for one hour at room temperature (RT), then incubated in primary antibody solution for 6 hours at RT plus overnight at $4\text{ }^{\circ}\text{C}$, then washed. Biotinylated secondary antibodies were applied for 2 hours at RT. They were anti-mouse Biotin-XX (for NET IR) or anti-rabbit Biotin-XX (for SERT IR). Where the serotonergic or noradrenergic afferents were double labelled together with either Zebrin II (mouse monoclonal antibody, Brochu et al, 1987) or aldolase C (rabbit polyclonal antibody, Sugihara and Shinoda, 2004), an Alexa-Fluor 594 coupled secondary antibody was added alongside the Biotinylated secondary (anti-mouse Alexa-Fluor 594 for Zebrin II and anti-rabbit Alexa-Fluor 594 for Aldolase C). Sections were washed and then received a tertiary treatment with Alexa-Fluor 488-conjugated Streptavidin. The tertiary incubations were for one hour at RT before a final set of washes (see Table 1 in Supplementary Material for a full details of all antibodies). Sections were mounted onto microscope slides in VECTASHIELD (Vector Laboratories) mounting medium and coverslipped.

200 μm sections were incubated in blocking solution overnight at RT, then incubated in primary antibody for 24 hours at RT and then washed. Secondary antibodies were applied for 6 hours at RT plus overnight

incubation at 4 °C. Sections were then washed and incubated in Alexa-Fluor 488-conjugated streptavidin for four hours at RT, then washed and mounted on microscope slides as above.

Confocal and multi-photon microscopy

For the 40 µm sections images were collected using an Olympus FV-1000 TIRF confocal laser scanning microscope and for the 200 µm sections images were collected using a Leica TCS SP8 MP (multiphoton) microscope. The multiphoton microscope allowed for deeper penetration of the excitation-laser in the thicker sections.

The long axes of individual cerebellar folia only run truly medial to lateral in the midline. Therefore, coronal sections are only strictly parallel to the medial-lateral axis at the midline. Similarly, parasagittal sections are only strictly transverse to this axis in the midline. For this reason, our analyses focused on regions at, and close to, the midline vermis of lobules IV, V and VI where the foliar curvature is least pronounced and where foliar and zonal/microzonal orientations are most easily defined. Using optical epifluorescence, the vermis lobules were first located in the sections. Multiple laser confocal image montages that encompassed a large region of the chosen lobule/ folium were collected using the tracking stage. Montages from coronal sections covered the whole width of the vermis of the lobule of interest. Parasagittal sections from one side of the midline vermis, through the midline and beyond to the other side were imaged and, for each section, the entire parasagittal extents of the lobule of interest were covered. All confocal images used for measurement of fibre extents were collected using a x20 objective giving a resolution of 0.625 µm per pixel. For coronal and parasagittal section images, each x-y image within the montage was itself a z-stack series at 0.8 µm steps through the entire depth of the section.

Image analysis and data analysis

All confocal digital images were analysed and prepared for presentation using Fiji/ ImageJ2 (Rueden et al. 2017; Schindelin et al. 2012).

For all coronal and parasagittal sections, a collapsed z-stack image was used to construct a smooth, continuous line through the centre of the Purkinje cell layer (PCL). At 300 µm intervals along this line, perpendiculars were constructed through the molecular layer (ML) to the pial surface. These perpendiculars and the PCL line were then projected down through the original z-stack to produce a set of transect plane surfaces perpendicular to

the ML and a Purkinje cell plane surface (yellow planes, Fig. 2A). For coronal sections, the perpendicular planes were transverse to the long axis of the folium. For sagittal sections, these planes were parallel to the long axis of the folium. These transect planes were used to provide an unbiased sample of the fibres in the ML. We measured only fibres crossing these ML transect planes. Every such fibre crossing a ML transect plane was selected and highlighted using the tracing tool Simple Neurite Tracer Plugin (Longair et al. 2011). If there were clear and minor discontinuities (typically 2 μm and never $> 5 \mu\text{m}$), they were connected. Any discontinuities of $> 5 \mu\text{m}$ were considered to be ends of fibres. This tracing routine left a permanent axon highlight identifier which ensured that any axon that crossed multiple perpendicular ML transect planes was measured only once.

The tracing through the z-stack generated lengths of ML noradrenergic and serotonergic fibres in three dimensions within the ML. This measure of the actual length of each fibre does not however indicate the rostral-caudal or medial-lateral folial extents because the fibre orientation may be either radial, oblique or with a tortuous path. Additionally, the folia have convexities or concavities as viewed from the surface, so afferent fibres of similar lengths can have interactions with different numbers of Purkinje cell dendrites within the molecular layer dependent upon the degree of curvature of the folium and their depth within the molecular layer. To measure fibre extents in planes relevant to the foliar axes, the underlying zonal architecture and to the Purkinje cells that are the output monolayer of the cerebellar cortex, we used the 3D trajectory analysis to measure the relationship of the fibre to its underlying Purkinje cells. At the two ends of each measured fibre, lines perpendicular to the PCL line were constructed and the distance between them along the PCL line was measured (see Fig. 2B). These measures were obtained in the coronal and parasagittal sections and for both fibre types to provide effective PCL extents (PCLE) in the coronal (medial-lateral) and parasagittal (rostral-caudal) planes.

All images presented in the Results section are of z-stack maximum intensity projections, as were used in the creation of the PCLE constructions.

Descriptive statistics are presented for PCLE fibre length measurements of SERT/40 μm /coronal, $n = 6$; SERT/40 μm /parasagittal, $n = 6$; SERT/200 μm /coronal, $n = 5$; NET/40 μm /coronal, $n = 6$; NET/40 μm /parasagittal, $n = 6$; NET/200 μm /coronal, $n = 5$. For planned group comparisons, the number of cases per group indicated the use of non-parametric tests and Mann-Whitney tests were used (SPSS 19, IBM). The group data were non-overlapping, so each test gave the maximum U-statistic of 0.

RESULTS

SERT and NET immunoreactive fibres (SERT⁺ and NET⁺, respectively) were observed in all three cortical layers (Fig. 3) and both fibre types were present across all regions of the cerebellar cortex. SERT⁺ and NET⁺ fibres display the distinctive varicosities (Fig. 3B and 3D, respectively) characteristic of monoaminergic 'beaded' fibres (Bishop and Ho 1985; Bishop et al. 1985; Chan-Palay 1975; Kerr and Bishop 1991; Powers et al. 1989; Takeuchi et al. 1982).

All afferent fibre trajectory lengths are described with reference to the underlying PC layer as PCLE values (see Methods) of mean, median and Standard Error of Mean (\bar{x} , \tilde{x} , SEM) in μm .

Serotonergic afferent distribution: 40 μm coronal sections

In coronal sections SERT⁺ fibres were seen to ascend perpendicularly, or slightly obliquely, to the PCL and pass through it into the ML where they either bifurcate or bend at approximately 90° to extend parallel to the PCL along the long axis of the folium for up to several hundred microns in the medial-lateral plane (Fig. 4A) (\bar{x} = 143.16, \tilde{x} = 152.54, SEM = 7.72, μm).

Approximately 10% of these SERT⁺ fibre PCLEs are >300 μm , 50% are in the range of 150-300 μm , and 40% are <150 μm (see Fig 4C for details). Overall, these trajectories are very similar to those of the granule cell ascending axon and parallel fibres. The small number of punctate SERT⁺ entities (Fig. 4A) suggest that very few SERT⁺ fibres pass perpendicular to the plane of section.

In the granule cell layer (GCL) many SERT⁺ fibres are short with a wide range of orientations. The small number of longer fibres that appear in the GCL are mostly oriented radially, probably as segments of fibres destined to terminate in the ML.

Serotonergic afferent distribution: 40 μm parasagittal sections

In parasagittal sections, the appearance of SERT⁺ fibres in the ML (Fig. 4B) is very different from that in coronal sections. Long fibres along the foliar axis are largely absent and the labelled fibres appear short or punctate. The PCLEs of these fibres are much shorter (\bar{x} = 30.58, \tilde{x} = 37.84, SEM = 7.74 μm) than those seen in coronal

sections with more than 90 % <150 μm (see Fig 4D for details). The distributions of PCLEs of SERT⁺ fibres in coronal and parasagittal sections are essentially non-overlapping; so the lengths in parasagittal sections are significantly smaller ($U = 0$, $n_1 = n_2 = 6$, $p < 0.01$. See Fig. 4E).

These data reveal a significant anisotropy in the distribution of serotonergic afferents in the cerebellar cortex; their trajectories are very similar to those of parallel fibres. Individual serotonergic fibres travel extensively in the medial-lateral plane, along the long axis of the folium, and deviate only very slightly in the rostral-caudal plane, transverse to the foliar axis.

Noradrenergic afferent distribution: 40 μm coronal sections

In coronal sections, NET⁺ fibres in ML at the midline vermis appear entirely different from SERT⁺ fibres in the same plane (see Fig. 5A). Most NET⁺ fibres are short or punctate, a small number are longer and radially-oriented and very few are long and along the folial axis. In this plane of section, only 1% of NET⁺ fibre PCLEs are >300 μm and 5% are in the range of 150-300 μm with the remaining 94% measuring <150 μm ; overall, approximately 35% of all NET⁺ fibres are <20 μm (Fig. 5C) ($\bar{x} = 53.64$, $\tilde{x} = 50.53$, $\text{SEM} = 3.27 \mu\text{m}$). Most NET⁺ fibres in the GCL and PCL are short or punctate but those that are radially-oriented are longer and extend towards the ML.

Noradrenergic afferent distribution: 40 μm parasagittal sections

In parasagittal sections, NET⁺ fibres have longer trajectories (Fig. 5B) than those in coronal sections (Fig. 5A). In general, these longer NET⁺ fibres perpendicular to the foliar axis are slightly more prevalent at the deeper levels of the ML, closer to the PCL, and they are less prevalent in the more superficial levels of ML than foliar axial SERT⁺ fibres (Compare Fig. 5B with Fig. 4A). PCLE measures reveal that these NET⁺ fibres are moderately long ($\bar{x} = 133.6$, $\tilde{x} = 130.27$, $\text{SEM} = 17.07 \mu\text{m}$) with around 15 % >300 μm and 50% in the range of 150-300 μm . The distributions of NET⁺ fibre PCLEs in parasagittal sections and coronal sections are essentially non-overlapping and significantly greater in the parasagittal plane ($U = 0$, $n_1 = n_2 = 6$, $p < 0.01$, see Fig. 5E).

Analysis of maximum fibre extents in 200 μm coronal sections

As discussed in the Methods, the length of fibre trajectories along the long axis of the folium may be underestimated in coronal sections when the folium passes out of the plane of section. To check potential

underestimation 40 μm sections, which could be greater for the SERT⁺ fibres, 200 μm coronal sections were also analysed.

Serotonergic afferent distribution: 200 μm coronal sections

As in the 40 μm sections, SERT⁺ fibres in 200 μm sections are predominantly parallel to the PCL and long (Fig. 6A), as revealed by PCLE analysis ($\bar{x} = 349.59$, $\tilde{x} = 331.3$, SEM = 16.09 μm). SERT⁺ fibre PCLEs in 200 μm coronal sections ($n = 5$) are significantly longer than in 40 μm sections ($U = 0.000$, $n_1 = 5$, $n_2 = 6$, $p < 0.01$, see Fig. 6C). The maximum fibre length is greater (898 μm and 757 μm , respectively) and there are higher proportions of long fibres (200/40 μm sections: 10/1% >600; 40/9% 300-600 μm ; 30/50% 150-300 μm . See Fig 6B). This comparison demonstrates that measurements made in 200 μm coronal sections better estimate the true lengths and therefore the range of influence of SERT⁺ fibres in the medial-lateral plane (Fig. 7B).

Noradrenergic afferent distribution: 200 μm coronal sections

In 200 μm and 40 μm coronal sections there is a similar mixture of horizontal, radial and punctate NET⁺ fibres (Fig. 6B). The mean PCLE of NET⁺ fibres in 200 μm sections is around 100 μm ($\bar{x} = 98.46$, $\tilde{x} = 93.85$, SEM = 7.5 μm). As is the case for SERT⁺ fibres, NET⁺ fibre PCLEs are significantly greater in the 200 μm sections ($U = 0$, $n_1 = n_2 = 6$, $p < 0.01$, see Fig. 6F). However, most of the fibres measured are still <150 μm , though the proportion of fibres >150 μm is increased (200/40 μm sections: 3/1 % >300 μm ; 20/5 % 150-300 μm ; 77/94 % <150 μm ; Fig. 6E).

These measurements from 200 μm coronal sections indicate that those from 40 μm sections slightly underestimate the length of NET⁺ fibres but even in 200 μm section analysis, 97% have PCLEs of less than 300 μm . Published accounts suggest microzone widths of approximately 100-300 μm in rat (Apps and Hawkes 2009) and cat (Andersson and Oscarsson 1978; Ekerot and Jömtell 2003; Ekerot and Jömtell 2001; Ekerot et al. 1991; Jömtell and Ekerot 2002; Jömtell and Ekerot 2003). Thus, the range of dimensions of cortical spread for individual NET⁺ fibres is similar to the reported dimensions of some cortical microzones.

Summary of anisotropies in serotonergic and noradrenergic fibres

Measurement of SERT⁺ and NET⁺ fibre lengths in coronal and parasagittal sections reveal strong anisotropies in the trajectories of both fibre types. For SERT⁺ fibres, trajectories with limited rostral-caudal extents and

substantial medial-lateral extents indicate that serotonergic afferents principally travel along the long-axis of the folium as do parallel fibres. For NET⁺ fibres, trajectories with extents significantly larger in the rostral-caudal than in the medial-lateral plane indicate that noradrenergic afferents travel transverse to the folium in approximate alignment with the well-known, parasagittal, olivo-cortical zones. In this way, these two monoaminergic afferent fibre systems have trajectories that are orthogonal to each other to form a grid-like pattern for possible modulatory influences in the molecular layer (see Fig. 7).

5-HT afferents: relationship to Zebrin II expression and transverse zones in 40 µm sections

In the rat anterior vermis, single zones have widths up to approximately 1 mm (Apps and Hawkes 2009; Jörntell et al. 2000; see also Oscarsson 1979; Oscarsson and Sjölund 1977 for measurements in cat) and the maximum length of SERT⁺ fibre measured here was ~0.9 mm. If this is a true upper-limit then the 5-HT projection could certainly cross multiple microzones (microzone width: ~100-300 µm) within a zone and may bridge at least two adjacent zones (see the Discussion for further analysis).

The zonal and microzonal organization of the cerebellar cortex is marked by the expression of specific molecules in subsets of Purkinje cells. One such molecule, Zebrin II (aldolase C) is expressed in sets of Purkinje cells that immunohistochemistry reveals as Zebrin II⁺ or Zebrin II⁻ longitudinal stripes. The stripes are highly consistent within and between species and there is clear relationship between the Zebrin II +/- stripes and zonal boundaries (Apps and Hawkes 2009; Cerminara et al. 2013; Jörntell et al. 2000; Pijpers et al. 2006; Sugihara and Shinoda 2004; Voogd and Ruigrok 2004) (see Fig. 8D). Because of their considerable lengths along the foliar axis, SERT⁺ fibres could in principle traverse multiple cortical zones and so we combined immunohistochemical labelling of Zebrin II (Brochu et al 1987) and aldolase C (Sugihara and Shinoda, 2004) and SERT to reveal the relationship between 5-HT afferent fibres and functional cerebellar compartments.

Zebrin II immunohistochemistry at midline vermis in lobules IV-VI (locations of the regions shown in Fig. 8A-C are mapped in Fig. 8D) enabled clear identification of several zones – A, AX, X (in lobule VI) B and A2. Multiple SERT⁺ fibres occupied two of these zones and two SERT⁺ fibres are shown with distribution across three zones (Fig. 8C). Since the total extents of these two fibres are approximately 600 µm and 900 µm and these lengths are likely towards the maximum measurable in 40 µm sections due to foliar curvature, it is possible that these or similar SERT⁺ fibres may cross even more zones than seen here.

DISCUSSION

Our demonstration of serotonergic and noradrenergic fibres in all three cerebellar cortical layers is consistent with earlier reports of their presence from studies using a range of techniques (Bishop and Ho 1985; Chan-Palay 1975; Felten et al. 1986; Hökfelt and Fuxe 1969; Kimoto et al. 1981; Pickel et al. 1974; Takeuchi et al. 1982). But here we have also shown for the first time that there are strong anisotropies in the distributions of serotonergic and noradrenergic fibres in the cortical molecular layer and that the major axes of orientations of the serotonergic and noradrenergic fibres are orthogonal to each other. In the molecular layer, individual serotonergic afferents run extensively in the medial-lateral plane but are highly restricted in their rostral-caudal extents whilst the opposite is true for noradrenergic fibres (distribution summarised in Fig. 9). Because glutamatergic afferents of the climbing fibre and mossy/parallel fibre systems also have orthogonal anisotropies, the organisation patterns of these monoaminergic afferents are likely to have important implications for understanding the roles of 5-HT and NA in cerebellar information processing.

The distributions of cerebellar serotonergic afferents and mossy /parallel fibre afferents have similarities.

Individual serotonergic fibres were revealed to extend over long distances medio-laterally, with many reaching up to 600 μm and some considerably longer. It should be noted that our estimates of serotonergic afferent lengths were limited by the application of planar imaging to the curved folia. Even 200 μm sections do not capture the complete curvature of a single folium, so total fibre lengths may be higher still.

Thus, most serotonergic afferents will cross multiple microzones, which have typical widths of 100 - 300 μm (Andersson and Oscarsson 1978; Apps and Hawkes 2009; Ekerot and Jömtell 2003; Ekerot and Jörintell 2001; Ekerot et al. 1991; Jörintell and Ekerot 2002; Jörintell and Ekerot 2003) and some can cross zones, as do the glutamatergic parallel fibres arising from granule cells. Mean parallel fibres lengths in these territories are 4.7mm and 4.2 mm for superficial and deep fibres, respectively with a small class of shorter parallel fibres with deep locations and with a range of lengths from 0.5mm to 1.1mm (Pichitpornchai et al 1994). The measured serotonergic fibre lengths are similar to those of this deep, short parallel fibre class. Further work free from planar imaging limitations could reveal whether they approach the lengths of the longer parallel fibre class.

Transzonal serotonergic afferent fibres: functional implications

Consistent with previous observations, serotonergic fibres were seen to have varicosities along their entire lengths and they are known to release 5-HT at these varicosities along their full extents. There are no

identified mechanisms by which release is differentially regulated across the varicosities to a propagated axonal depolarization, so a single 5-HT afferent is likely to release from all varicosities and so modulate activity in all microzones and zones that it traverses. That property is not well-suited to the selective modulation of an individual microzone or a specified set of non-adjacent microzones. Modulation of a set of heterogeneous microzones by serotonergic afferents might be useful in binding the activity of multiple effectors in a single action, as has been proposed by Schweighofer et al. (2004), but the afferent distributions we have seen are unlikely to support it. Instead, their strongly medial-lateral arrangement and their long extents are consistent with the suggestion that these fibres are more suited to providing a common signal to microzones identified by their contiguity rather than any functional relationships.

At present, there are few empirical studies that provide insights into the functional role of serotonergic afferents to the cerebellum at a behavioural level. Some have investigated 5-HT function in a model form of cerebellum-dependent learning, the acquisition of nictitating membrane response conditioning in rabbits (see Harvey, 2003 for a review). Activation of 5-HT_{2A} receptors by systemic application of selective ligands enhanced rates of learning but conclusions about mechanism must be limited because the drugs would have affected not only the cerebellum but also a wide range of extracerebellar targets.

An earlier view that the Raphe nuclei are a major source of serotonergic afferents to the cerebellum is not supported by later empirical evidence because the substantial Raphe nuclei projections to the cerebellum do not co-localise with tryptophan hydroxylase (Bishop and Ho 1985; Kerr and Bishop 1991; Walker et al. 1988). The sources of serotonergic afferents to the cerebellar cortex are diverse and include a number of brainstem regions and nuclei in the rat and cat (Bishop and Ho 1985; Kerr and Bishop 1991, respectively). Kerr and Bishop (1991) showed serotonergic cerebellar innervation from the lateral reticular nuclei (LRN), lateral tegmental field (LTF) and paramedian reticular nuclei (PRN). Combined tracing and immunohistochemistry revealed that, within the LRN, LTF and PRN, only a small proportion of afferents are serotonergic together with a much larger population of non-serotonergic afferents, likely glutamatergic MFs. Via synaptic relay in the granule cell layer, information from these MF inputs is then distributed within the molecular layer by the medial-lateral PF system (see Fig. 1B). In principle, the medial-lateral distribution of information via these serotonergic and glutamatergic MF/PF systems means that related information from several brainstem regions can reach a cerebellar cortical territory via both afferent systems (Dieudonné, 2001) but the presence and functional implications of this dual representation, with opportunities for additional coincidence detection, are currently unknown.

Single cerebellar noradrenergic afferents map to small molecular layer territories

From our planar analyses of 40 μm sections, the noradrenergic afferent trajectories occupied small patches of molecular layer territory with a mean medial-lateral width of around 50 μm and a mean rostral-caudal extent of around 130 μm but with a large range of values up to 600 μm and more. The analysis of 200 μm coronal sections revealed mean medial-lateral widths of around 100 μm , so the planar analysis of 40 μm parasagittal sections could have underestimated the rostral-caudal extents of some these fibres. Nonetheless, the molecular layer patches occupied by fibres are clearly longer in the rostral-caudal direction with spatial extents similar to the dimensions of a cortical microzone. One exciting implication of this arrangement is that a single NA afferent to the molecular layer could modulate function in an individual microzone. Thus, groups of functionally associated but non-adjacent microzones could be modulated by patterned activity in the locus coeruleus (LC) or other noradrenergic precerebellar source nuclei (Robertson et al. 2013).

Functional implications of single noradrenergic afferent supply to small molecular layer territories

The suggestion that NA afferents might control individual microzones or specified sets of microzones would contrast with a contemporary view of the NA network in which afferents originating in the locus coeruleus transmit a broad signal that would regulate a more global function such as directed attention or modulation of behavioural arousal (Aston-Jones and Cohen 2005; Berridge and Waterhouse 2003; Bouret and Sara 2005; Sara and Bouret 2012). This view of the NA network was influenced by earlier observations that a comparatively small number of NA neurons in the LC innervate much of the brain and that a single neuron in the LC can supply two or more brain structures. Nagai et al. (1981) and Steindler (1981) found that significant proportions of LC neurons project both to the cerebellum and also to one of a number of forebrain structures. A recent study (Schwarz et al. 2015) using viral-genetic tracing found that many individual LC noradrenergic neurons project to a large number of target structures but that a small number of LC neurons have much more specific projection targets.

However, a growing body of evidence suggests that the functional organisation of the LC may be more complex. Fallon and Loughlin (1982) showed two populations of LC neurons: the LC core contains neurons with axons that collateralize many times, innervating multiple divergent brain structures, but the LC periphery contains neurons with fewer collaterals and target fields. Tracing from individual forebrain sub-regions has also revealed organization within the LC. LC afferents to the orbitofrontal, medial prefrontal, anterior cingulate and primary motor cortices arise from quite discrete and separate sets of LC neurons (Chandler and Waterhouse 2012; Chandler et al. 2014a; Chandler et al. 2013; Chandler et al. 2014b). Using a novel mRNA-based tracing technique Kobschull et al. (2016) traced the projection patterns of very large numbers of

individual noradrenergic LC neurons simultaneously. Each LC neuron has a specific preferred target in the cortex or olfactory bulb, though its collaterals also provide weaker innervation of other regions. Other tracing methods may not differentiate between these strong and weak innervations. Similar fine-grain analyses of organisation within the LC projection to the cerebellum are not yet available, but the parasagittal patterning of individual noradrenergic afferents in the molecular layer suggests that there could be value to heterogeneity in these noradrenergic inputs.

Recent studies have also demonstrated a previously unappreciated complexity in the activity of the LC itself. Multiple single unit recordings by Totah et al. (2018) revealed that only a small number of LC neurons exhibited simultaneous spontaneous and salient stimulus-evoked discharges, in contrast to earlier studies suggesting concerted LC activity, particularly in response to salient stimuli (Aston-Jones and Bloom 1981). Aversion learning and behavioural flexibility (Uematsu et al. 2017) and nociception and aversion behaviour (Hirschberg et al. 2017) engage non-overlapping populations of LC neurons that are specifically distributed, exhibit task-dependent response properties and make distinct contributions to behaviour.

Thus, there is growing evidence that activity in LC neurons is heterogeneous and that their outputs are regionally selective. Our finding that each noradrenergic afferent occupies a small territory within the molecular layer that may correspond to a functional microzone suggests that noradrenergic modulation of cerebellar cortical function may be fine grain. But with a mere 3200 noradrenergic neurons in the LC of the rat (Sara and Bouret 2012), and a considerably larger number of noradrenergic fibres in the molecular layer of our analysed material, further analysis will be needed to understand whether the information content of the noradrenergic system is distributed within the cerebellum at the microzonal level.

In summary, the two most significant neuromodulatory afferents to the cerebellar cortex display contrasting arrangements of their fibres that indicate they probably fulfil distinct roles within the cerebellar cortical network. The arrangement of the 5-HT afferent fibres is similar in several respects to that of PFs that travel along the long axis of the folium and can cross multiple cortical zones. This distribution suggests that the 5-HT afferents may provide a diffuse signal to multiple, functionally-independent regions simultaneously. In contrast, the arrangement of NA afferent fibres is indicative of a far more targeted signalling role in which NA afferent fibres may selectively modulate the activity of a small cortical territory, perhaps as discrete as a single microzone, a fundamental functional unit of the cerebellar cortex.

ACKNOWLEDGMENTS

This work was supported by a BBSRC studentship (ML). We thank Richard Hawkes for his gift of the anti-Zebrin II mouse monoclonal antibody and for his comments on the manuscript. We thank Izumi Sugihara for his gift of the anti-Aldolase C rabbit polyclonal antibody.

REFERENCES

- Albus JS (1971) A theory of cerebellar function *Mathematical Biosciences* 10:25-61 doi: 10.1016/0025-5564(71)90051-4
- Andersson G, Oscarsson O (1978) Climbing fiber microzones in cerebellar vermis and their projection to different groups of cells in the lateral vestibular nucleus *Experimental brain research* 32:565-579 doi:10.1007/BF00239553
- Apps R, Garwicz M (2005) Anatomical and physiological foundations of cerebellar information processing *Nat Rev Neurosci* 6:297-311 doi:10.1038/nrn1646
- Apps R, Hawkes R (2009) Cerebellar cortical organization: a one-map hypothesis *Nat Rev Neurosci* 10:670-681 doi:10.1038/nrn2698
- Aston-Jones G, Bloom FE (1981) Nonrepinephrine-containing locus coeruleus neurons in behaving rats exhibit pronounced responses to non-noxious environmental stimuli *The Journal of Neuroscience* 1:887 doi:10.1523/JNEUROSCI.01-08-00887.1981
- Aston-Jones G, Cohen JD (2005) An Integrative Theory of Locus Coeruleus-Norepinephrine Function: Adaptive Gain and Optimal Performance *Annual review of neuroscience* 28:403-450 doi:doi:10.1146/annurev.neuro.28.061604.135709
- Beaudet A, Sotelo C (1981) Synaptic remodeling of serotonin axon terminals in rat agranular cerebellum *Brain research* 206:305-329 doi: 10.1016/0006-8993(81)90534-5
- Berridge CW, Waterhouse BD (2003) The locus coeruleus–noradrenergic system: modulation of behavioral state and state-dependent cognitive processes *Brain Research Reviews* 42:33-84 doi: 10.1016/S0165-0173(03)00143-7
- Bishop GA, Ho RH (1985) The distribution and origin of serotonin immunoreactivity in the rat cerebellum *Brain research* 331:195-207 doi: 10.1016/0006-8993(85)91545-8
- Bishop GA, Ho RH, King JS (1985) Localization of serotonin immunoreactivity in the opossum cerebellum *J Comp Neurol* 235:301-321 doi:10.1002/cne.902350303
- Bloom FE, Hoffer BJ, Siggins GR (1971) Studies on norepinephrine-containing afferents to Purkinje cells of rat cerebellum. I. Localization of the fibers and their synapses *Brain research* 25:501-521 doi: 10.1016/0006-8993(71)90457-4

- Bouret S, Sara SJ (2005) Network reset: a simplified overarching theory of locus coeruleus noradrenaline function *Trends Neurosci* 28:574-582 doi:10.1016/j.tins.2005.09.002
- Buisseret-Delmas C, Angaut P (1993) The cerebellar olivo-corticonuclear connections in the rat *Progress in Neurobiology* 40:63-87 doi: 10.1016/0301-0082(93)90048-W
- Cerminara NL, Aoki H, Loft M, Sugihara I, Apps R (2013) Structural basis of cerebellar microcircuits in the rat *The Journal of neuroscience : the official journal of the Society for Neuroscience* 33:16427-16442 doi:10.1523/JNEUROSCI.0861-13.2013
- Chan-Palay V (1975) Fine structure of labelled axons in the cerebellar cortex and nuclei of rodents and primates after intraventricular infusions with tritiated serotonin *Anat Embryol* 148:235-265 doi:10.1007/BF00319846
- Chandler D, Waterhouse BD (2012) Evidence for broad versus segregated projections from cholinergic and noradrenergic nuclei to functionally and anatomically discrete subregions of prefrontal cortex *Frontiers in behavioral neuroscience* 6:20 doi:10.3389/fnbeh.2012.00020
- Chandler DJ, Gao WJ, Waterhouse BD (2014a) Heterogeneous organization of the locus coeruleus projections to prefrontal and motor cortices *Proceedings of the National Academy of Sciences of the United States of America* 111:6816-6821 doi:10.1073/pnas.1320827111
- Chandler DJ, Lamperski CS, Waterhouse BD (2013) Identification and distribution of projections from monoaminergic and cholinergic nuclei to functionally differentiated subregions of prefrontal cortex *Brain research* 1522:38-58 doi:10.1016/j.brainres.2013.04.057
- Chandler DJ, Waterhouse BD, Gao WJ (2014b) New perspectives on catecholaminergic regulation of executive circuits: evidence for independent modulation of prefrontal functions by midbrain dopaminergic and noradrenergic neurons *Front Neural Circuits* 8:53 doi:10.3389/fncir.2014.00053
- Coutinho V, Mutoh H, Knopfel T (2004) Functional topology of the mossy fibre-granule cell--Purkinje cell system revealed by imaging of intrinsic fluorescence in mouse cerebellum *The European journal of neuroscience* 20:740-748 doi:10.1111/j.1460-9568.2004.03533.x
- Dean I, Robertson SJ, Edwards FA (2003) Serotonin Drives a Novel GABAergic Synaptic Current Recorded in Rat Cerebellar Purkinje Cells: A Lugaro Cell to Purkinje Cell Synapse *The Journal of Neuroscience* 23:4457 doi:10.1523/JNEUROSCI.23-11-04457.2003
- Dean P, Porrill J, Ekerot C-F, Jorntell H (2010) The cerebellar microcircuit as an adaptive filter: experimental and computational evidence *Nat Rev Neurosci* 11:30-43 doi:http://www.nature.com/nrn/journal/v11/n1/supinfo/nrn2756_S1.html
- Di Mauro M, Fretto G, Caldera M, Li Volsi G, Licata F, Ciranna L, Santangelo F (2003) Noradrenaline and 5-hydroxytryptamine in cerebellar nuclei of the rat: functional effects on neuronal firing *Neuroscience Letters* 347:101-105 doi: 10.1016/S0304-3940(03)00509-3
- Dieudonné S (2001) Serotonergic Neuromodulation in the Cerebellar Cortex: Cellular, Synaptic, and Molecular Basis *The Neuroscientist* 7:207-219 doi.org/10.1177/107385840100700306
- Dieudonné S, Dumoulin A (2000) Serotonin-Driven Long-Range Inhibitory Connections in the Cerebellar Cortex *The Journal of Neuroscience* 20:1837 doi:10.1523/JNEUROSCI.20-05-01837.2000

- Eccles JC, Ito M, Szentágothai Jn (1967) The cerebellum as a neuronal machine. Springer-Verlag, Berlin, New York etc.
- Ekerot C-F, Jömtell H (2003) Parallel fiber receptive fields: a key to understanding cerebellar operation and learning *The Cerebellum* 2:101 doi:10.1080/14734220309411
- Ekerot C-F, Jömtell H (2001) Parallel fibre receptive fields of Purkinje cells and interneurons are climbing fibre-specific *European Journal of Neuroscience* 13:1303-1310 doi:10.1046/j.0953-816x.2001.01499.x
- Ekerot CF, Garwicz M, Schouenborg J (1991) Topography and nociceptive receptive fields of climbing fibres projecting to the cerebellar anterior lobe in the cat *The Journal of Physiology* 441:257-274 doi:10.1113/jphysiol.1991.sp018750
- Ekerot CF, Larson B (1979a) The dorsal spino-olivocerebellar system in the cat. I. Functional organization and termination in the anterior lobe *Experimental brain research* 36:201-217 doi:10.1007/BF00238905
- Ekerot CF, Larson B (1979b) The dorsal spino-olivocerebellar system in the cat. II. Somatotopical organization *Experimental brain research* 36:219-232 doi:10.1007/BF00238906
- Fallon JH, Loughlin SE (1982) Monoamine innervation of the forebrain: Collateralization *Brain Research Bulletin* 9:295-307 doi: 10.1016/0361-9230(82)90143-5
- Felten DL, Felten SY, Perry KW, Fuller RW, Nurnberger JI, Ghetti B (1986) Noradrenergic innervation of the cerebellar cortex in normal and in Purkinje cell degeneration mutant mice: Evidence for long term survival following loss of the two major cerebellar cortical neuronal populations *Neuroscience* 18:783-793 doi: 10.1016/0306-4522(86)90099-0
- Garwicz M, Ekerot CF (1994) Topographical organization of the cerebellar cortical projection to nucleus interpositus anterior in the cat vol 474. doi:10.1113/jphysiol.1994.sp020017
- Geurts FJ, De Schutter E, Timmermans J-P (2002) Localization of 5-HT_{2A}, 5-HT₃, 5-HT_{5A} and 5-HT₇ receptor-like immunoreactivity in the rat cerebellum *Journal of Chemical Neuroanatomy* 24:65-74 doi: 10.1016/S0891-0618(02)00020-0
- Gilbert PFC (1975) How the cerebellum could memorise movements *Nature* 254:688-689 doi:10.1038/254688a0
- Gilbert PFC (1974) A theory of memory that explains the function and structure of the cerebellum *Brain research* 70:1-18 doi: 10.1016/0006-8993(74)90208-X
- Harvey JA (2003) Role of the serotonin 5-HT_{2A} receptor in learning *Learning & memory* 10:355-362 doi:10.1101/lm.60803
- Harvey RJ, Napper RM (1988) Quantitative study of granule and Purkinje cells in the cerebellar cortex of the rat *J Comp Neurol* 274:151-157 doi:10.1002/cne.902740202
- Hesslow G (1994a) Correspondence between climbing fibre input and motor output in eyeblink-related areas in cat cerebellar cortex *The Journal of physiology* 476:229-244 doi: 10.1113/jphysiol.1994.sp020126
- Hesslow G (1994b) Inhibition of classically conditioned eyeblink responses by stimulation of the cerebellar cortex in the decerebrate cat *The Journal of Physiology* 476:245-256 doi:10.1113/jphysiol.1994.sp020127

- Hirono M, Matsunaga W, Chimura T, Obata K (2008) Developmental enhancement of alpha2-adrenoceptor-mediated suppression of inhibitory synaptic transmission onto mouse cerebellar Purkinje cells *Neuroscience* 156:143-154 doi:10.1016/j.neuroscience.2008.07.018
- Hirschberg S, Li Y, Randall A, Kremer EJ, Pickering AE (2017) Functional dichotomy in spinal- vs prefrontal-projecting locus coeruleus modules splits descending noradrenergic analgesia from ascending aversion and anxiety in rats *eLife* 6:e29808 doi:10.7554/eLife.29808
- Hökfelt T, Fuxe K (1969) Cerebellar monoamine nerve terminals, a new type of afferent fibers to the cortex cerebelli *Experimental brain research* 9:63-72 doi:10.1007/BF00235452
- Ito M (1982) Cerebellar control of the vestibulo-ocular reflex--around the flocculus hypothesis *Annual review of neuroscience* 5:275-296 doi:10.1146/annurev.ne.05.030182.001423
- Ito M (1984) *The Cerebellum and Neural Control*. Raven Press, New York
- Jörntell H, Ekerot C, Garwicz M, Luo XL (2000) Functional organization of climbing fibre projection to the cerebellar anterior lobe of the rat *The Journal of physiology* 522 Pt 2:297-309 doi:10.1111/j.1469-7793.2000.00297.x
- Jörntell H, Ekerot C-F (2002) Reciprocal Bidirectional Plasticity of Parallel Fiber Receptive Fields in Cerebellar Purkinje Cells and Their Afferent Interneurons *Neuron* 34:797-806 doi:10.1016/S0896-6273(02)00713-4
- Jörntell H, Ekerot C-F (2003) Receptive Field Plasticity Profoundly Alters the Cutaneous Parallel Fiber Synaptic Input to Cerebellar Interneurons In Vivo *The Journal of Neuroscience* 23:9620 doi:10.1523/JNEUROSCI.23-29-09620.2003
- Kebschull JM, Garcia da Silva P, Reid AP, Peikon ID, Albeanu DF, Zador AM (2016) High-Throughput Mapping of Single-Neuron Projections by Sequencing of Barcoded RNA *Neuron* 91:975-987 doi:10.1016/j.neuron.2016.07.036
- Kerr CW, Bishop GA (1991) Topographical organization in the origin of serotonergic projections to different regions of the cat cerebellar cortex *J Comp Neurol* 304:502-515 doi:10.1002/cne.903040313
- Kimoto Y, Tohyama M, Satoh K, Sakumoto T, Takahashi Y, Shimizu N (1981) Fine structure of rat cerebellar noradrenaline terminals as visualized by potassium permanganate 'in situ perfusion' fixation method *Neuroscience* 6:47-58 doi: 10.1016/0306-4522(81)90242-6
- Kitzman PH, Bishop GA (1994) The origin of serotonergic afferents to the cat's cerebellar nuclei *J Comp Neurol* 340:541-550 doi:10.1002/cne.903400407
- Landis SC, Bloom FE (1975) Ultrastructural identification of noradrenergic boutons in mutant and normal mouse cerebellar cortex *Brain research* 96:299-305 doi: 10.1016/0006-8993(75)90738-6
- Landis SC, Shoemaker WJ, Schlumpf M, Bloom FE (1975) Catecholamines in mutant mouse cerebellum: Fluorescence microscopic and chemical studies *Brain research* 93:253-266 doi: 10.1016/0006-8993(75)90349-2
- Longair MH, Baker DA, Armstrong JD (2011) Simple Neurite Tracer: open source software for reconstruction, visualization and analysis of neuronal processes *Bioinformatics* 27:2453-2454 doi:10.1093/bioinformatics/btr390

- Marr D (1969) A theory of cerebellar cortex *The Journal of Physiology* 202:437-470
doi:10.1113/jphysiol.1969.sp008820
- Miles FA, Lisberger SG (1981) Plasticity in the vestibulo-ocular reflex: a new hypothesis *Annual review of neuroscience* 4:273-299 doi:10.1146/annurev.ne.04.030181.001421
- Mugnaini E, Dahl AL (1975) Mode of distribution of aminergic fibers in the cerebellar cortex of the chicken *J Comp Neurol* 162:417-432 doi:10.1002/cne.901620402
- Nagai T, Satoh K, Imamoto K, Maeda T (1981) Divergent projections of catecholamine neurons of the locus coeruleus as revealed by fluorescent retrograde double labeling technique *Neuroscience Letters* 23:117-123 doi: 10.1016/0304-3940(81)90027-6
- Napper RM, Harvey RJ (1988) Number of parallel fiber synapses on an individual Purkinje cell in the cerebellum of the rat *J Comp Neurol* 274:168-177 doi:10.1002/cne.902740204
- Nielsen K, Brask D, Knudsen GM, Aznar S (2006) Immunodetection of the serotonin transporter protein is a more valid marker for serotonergic fibers than serotonin *Synapse* 59:270-276 doi:10.1002/syn.20240
- Oscarsson O (1979) Functional units of the cerebellum - sagittal zones and microzones *Trends in Neurosciences* 2:143-145 doi:10.1016/0166-2236(79)90057-2
- Oscarsson O, Sjölund B (1977) The ventral spino-olivocerebellar system in the cat. II. Termination zones in the cerebellar posterior lobe *Experimental brain research* 28:487-503 doi:10.1007/BF00236472
- Papay R et al. (2006) Localization of the mouse alpha1A-adrenergic receptor (AR) in the brain: alpha1AAR is expressed in neurons, GABAergic interneurons, and NG2 oligodendrocyte progenitors *J Comp Neurol* 497:209-222 doi:10.1002/cne.20992
- Papay R, Gaivin R, McCune DF, Rorabaugh BR, Macklin WB, McGrath JC, Perez DM (2004) Mouse alpha1B-adrenergic receptor is expressed in neurons and NG2 oligodendrocytes *J Comp Neurol* 478:1-10 doi:10.1002/cne.20215
- Paschalis A, Churchill L, Marina N, Kasymov V, Gourine A, Ackland G (2009) beta1-Adrenoceptor distribution in the rat brain: an immunohistochemical study *Neurosci Lett* 458:84-88
doi:10.1016/j.neulet.2009.04.023
- Pichitpornchai C, Rawson JA, Rees S (1994) Morphology of parallel fibres in the cerebellar cortex of the rat: an experimental light and electron microscopic study with biocytin *J Comp Neurol* 342:206-220
doi:10.1002/cne.903420205
- Pickel VM, Segal M, Bloom FE (1974) A radioautographic study of the efferent pathways of the nucleus locus coeruleus *J Comp Neurol* 155:15-42 doi:10.1002/cne.901550103
- Pijpers A, Apps R, Pardoe J, Voogd J, Ruigrok TJ (2006) Precise spatial relationships between mossy fibers and climbing fibers in rat cerebellar cortical zones *The Journal of neuroscience : the official journal of the Society for Neuroscience* 26:12067-12080 doi:10.1523/JNEUROSCI.2905-06.2006
- Porrill J, Dean P, Anderson SR (2013) Adaptive filters and internal models: Multilevel description of cerebellar function *Neural Networks* 47:134-149 doi: 10.1016/j.neunet.2012.12.005
- Powers RE, O'Connor DT, Price DL (1989) Noradrenergic systems in human cerebellum *Brain research* 481:194-199 doi: 10.1016/0006-8993(89)90504-0

- Qian Y, Melikian HE, Rye DB, Levey AI, Blakely RD (1995) Identification and characterization of antidepressant-sensitive serotonin transporter proteins using site-specific antibodies *The Journal of Neuroscience* 15:1261 doi:10.1523/JNEUROSCI.15-02-01261.1995
- Robertson SD, Plummer NW, de Marchena J, Jensen P (2013) Developmental origins of central norepinephrine neuron diversity *Nat Neurosci* 16:1016-1023 doi:10.1038/nn.3458
- Rueden CT, Schindelin J, Hiner MC, DeZonia BE, Walter AE, Arena ET, Eliceiri KW (2017) ImageJ2: ImageJ for the next generation of scientific image data *BMC bioinformatics* 18:529-529 doi:10.1186/s12859-017-1934-z
- Sara SJ, Bouret S (2012) Orienting and reorienting: the locus coeruleus mediates cognition through arousal *Neuron* 76:130-141 doi:10.1016/j.neuron.2012.09.011
- Schindelin J et al. (2012) Fiji: an open-source platform for biological-image analysis *Nat Meth* 9:676-682 doi:<http://www.nature.com/nmeth/journal/v9/n7/abs/nmeth.2019.html#supplementary-information>
- Schroeter S, Apparsundaram S, Wiley RG, Miner LH, Sesack SR, Blakely RD (2000) Immunolocalization of the cocaine- and antidepressant-sensitive l-norepinephrine transporter *Journal of Comparative Neurology* 420:211-232 doi:10.1002/(SICI)1096-9861(20000501)420:2<211::AID-CNE5>3.0.CO;2-3
- Schwarz LA et al. (2015) Viral-genetic tracing of the input-output organization of a central noradrenaline circuit *Nature* 524:88-92 doi:10.1038/nature14600
- Schweighofer N, Doya K, Kuroda S (2004) Cerebellar aminergic neuromodulation: towards a functional understanding *Brain Research Reviews* 44:103-116 doi:10.1016/j.brainresrev.2003.10.004
- Steindler DA (1981) Locus coeruleus neurons have axons that branch to the forebrain and cerebellum *Brain research* 223:367-373 doi: 10.1016/0006-8993(81)91149-5
- Sugihara I, Shinoda Y (2004) Molecular, topographic, and functional organization of the cerebellar cortex: a study with combined aldolase C and olivocerebellar labeling *The Journal of neuroscience : the official journal of the Society for Neuroscience* 24:8771-8785 doi:10.1523/JNEUROSCI.1961-04.2004
- Sur C, Betz H, Schloss P (1996) Immunocytochemical detection of the serotonin transporter in rat brain *Neuroscience* 73:217-231 doi: 10.1016/0306-4522(96)00030-9
- Takeuchi Y, Kimura H, Sano Y (1982) Immunohistochemical demonstration of serotonin-containing nerve fibers in the cerebellum *Cell and Tissue Research* 226:1-12 doi:10.1007/BF00217077
- Totah NK, Neves RM, Panzeri S, Logothetis NK, Eschenko O (2018) The Locus Coeruleus Is a Complex and Differentiated Neuromodulatory System *Neuron* 99:1055-1068.e1056 doi:10.1016/j.neuron.2018.07.037
- Uematsu A et al. (2017) Modular organization of the brainstem noradrenaline system coordinates opposing learning states *Nature Neuroscience* 20:1602 doi:10.1038/nn.4642
- Verney C, Grzanna R, Farkas E (1982) Distribution of Dopamine-Beta-Hydroxylase-Like Immunoreactive Fibers in the Rat Cerebellar Cortex During Ontogeny *Developmental Neuroscience* 5:369-374 doi:10.1159/000112788

- Voogd J, Bigare F (1980) Topographical distribution of olivary and cortico nuclear fibres in the cerebellum: a review. In: Courville J, de Montigny C, Lamarre Y (eds) *The Inferior Olivary Nucleus Anatomy and Physiology*. Raven, New York, pp 207-234
- Voogd J, Ruigrok TJ (2004) The organization of the corticonuclear and olivocerebellar climbing fiber projections to the rat cerebellar vermis: the congruence of projection zones and the zebrin pattern *J Neurocytol* 33:5-21 doi:10.1023/B:NEUR.0000029645.72074.2b
- Walker JJ, Bishop GA, Ho RH, King JS (1988) Brainstem origin of serotonin- and enkephalin-immunoreactive afferents to the opossum's cerebellum *The Journal of Comparative Neurology* 276:481-497 doi:10.1002/cne.902760403
- Yamamoto T, Ishikawa M, Tanaka C (1977) Catecholaminergic terminals in the developing and adult rat cerebellum *Brain research* 132:355-361 doi: 10.1016/0006-8993(77)90428-0
- Yeo CH, Hesslow G (1998) Cerebellum and conditioned reflexes *Trends Cogn Sci* 2:322-330 doi: 10.1016/S1364-6613(98)01219-4
- Zhang J, Muller JF, McDonald AJ (2013) Noradrenergic innervation of pyramidal cells in the rat basolateral amygdala *Neuroscience* 228:395-408 doi:10.1016/j.neuroscience.2012.10.035 *System Neuron* 99:1055-1068.e1056 doi:10.1016/j.neuron.2018.07.037

FIGURE LEGENDS

Fig 1. Organization of cerebellar longitudinal zones and afferent fibre orientations in the cerebellar cortex.

A: Organisation of glutamatergic afferents to Purkinje cells (PC - blue). Mossy fibre afferents (MF- black) synapse upon granule cells, whose axons ascend and then project widely in the medial-lateral direction as parallel fibres (PF - orange) passing through and making synaptic contact with large numbers of different Purkinje cell dendritic trees. Climbing fibres (CF - red) contact PCs in a one-to-one manner; climbing fibres from the same olivary sub-nucleus terminate in a restricted parasagittal territory giving rise to a longitudinal zone.

B: The positions of the longitudinal zones and transverse lobules in the rat cerebellar cortex on a simplified, flattened map of Lobules IV to VII. The figure is based on published data (Voogd and Ruigrok, 2000; Jorntell et al, 2000; Sugihara and Shinoda, 2004; Voogd and Ruigrok, 2004; Pijpers et al, 2006; Sugihara et al, 2009; Cerminara et al, 2013). Abbreviations: M: Medial, L: Lateral, R: Rostral, C: Caudal

Fig 2. Sampling and measurement processes for PCLE analysis. **A:** Transect planes, spaced at 300 μm intervals were projected through the Z-plane to sample all fibres crossing them at any height or depth in the section. **B:** A PCL midline was also projected through the Z-plane. The traced fibres were measured on a collapsed projection of the z-stack. Two lines were projected down and perpendicular to the PCL midline from the ends of each measured fibre. The proposed functional extent of the fibre on the PCL midline was measured as the PCL extent (PCLE).

Fig 3. SERT⁺ and NET⁺ fibres are present throughout the cerebellar cortex. SERT⁺ (A,B) and NET⁺ (C, D) fibres are present in all three layers of the cerebellar cortex: the molecular layer (ML), Purkinje cell layer (PCL) and granule cell layer (GCL). Characteristic monoaminergic ‘beaded’ fibres are revealed with anti-SERT (B) and anti-NET (D) antibodies (examples highlighted within dotted line ellipses). All images of 40 μm sections, scale bars 50 μm (A, C) and 25 μm (B, D).

Fig 4. SERT⁺ fibres project extensively in the medial-lateral plane but their trajectories are restricted in the rostral-caudal plane. Analysis of 40 μm sections. **A:** Coronal section. In this section plane many SERT⁺ fibres extend for long distances medio-laterally in the molecular layer (blue and pink highlight two examples). Radially oriented fibres (pointers show two examples) and a small number of punctate fibres (example circled) are also seen in coronal sections (scale bar: 150 μm). **B:** Parasagittal section. In this section plane, the majority of molecular layer SERT⁺ fibres are punctate or very short; they have restricted rostral-caudal projections (scale bar: 150 μm). **C and D:** Distribution of SERT⁺ fibre PCLEs (100 μm bins) in coronal and parasagittal sections respectively. **E:** Comparison of means (± 1 SEM) of SERT⁺ fibre PCLEs for both section orientations.

Fig 5. NET⁺ fibres have restricted medial-lateral projections. Analysis of 40 μm sections. **A:** In coronal sections, the majority of NET⁺ fibres in the molecular layer are short or punctate (examples circled). There is a small number of radial fibres (pointer shows one example) and longer horizontal fibres (pink highlights two examples). Thus, NET⁺ fibres have restricted medial-lateral extents. Scale bar: 200 μm . **B:** In parasagittal sections, long NET⁺ fibres are common (pink highlights two examples) revealing that their trajectories are extensive in the rostral-caudal plane. Punctate fibres are scarce (example circled) Scale bar: 200 μm . **C and D:**

Distribution of NET⁺ fibre PCLEs (100 μm bins) in coronal and parasagittal sections, respectively. **E:** Mean (± 1 SEM) of NET⁺ PCLEs measured in both section orientations.

Fig 6. 200 μm coronal sections better estimate SERT⁺ and NET⁺ fibre lengths in the medial-lateral plane. **A:** In 200 μm coronal sections, long horizontal fibres (examples highlighted) are still the predominant SERT⁺ fibre type. Scale bar: 200 μm . **B:** Distribution of SERT⁺ fibre PCLEs (100 μm bins) in 200 μm coronal sections. **C:** Mean (± 1 SEM) of SERT⁺ fibre PCLEs in 200 μm and 40 μm sections. **D:** Higher number of longer horizontal NET⁺ fibre PCLEs measured in 200 μm coronal sections than in 40 μm sections (examples highlighted, scale bar: 200 μm) and low numbers of short or punctate fibres. **E:** Distribution of NET⁺ fibre PCLEs (100 μm bins) in 200 μm coronal sections. **F:** Mean (± 1 SEM) of NET⁺ fibre PCLEs in 200 μm and 40 μm sections.

Fig 7. Mean PCLEs reveal significant anisotropy in SERT⁺ and NET⁺ afferent fibre trajectories. **A:** PCLEs in the rostral-caudal and medial-lateral planes for SERT⁺ (green) and NET⁺ (red) fibres measured in 40 μm sections. **B:** PCLEs in the rostral-caudal and medial-lateral planes for SERT⁺ (green) and NET⁺ (red) fibres measured in 40 μm parasagittal sections and in 200 μm coronal sections. The 200 μm coronal sections captured more extended medial-lateral trajectories by partially compensating for foliar curvature away from the plane of section. The directional anisotropies seen for SERT⁺ and NET⁺ afferent fibres are orthogonal and significant in analyses of both section thicknesses.

Fig 8. SERT⁺ fibres bridge zonal boundaries identified by Zebrin II immunohistochemistry. SERT⁺ fibres (green fluorochrome) are seen to cross putative zones (yellow dashed lines and text) identified on the basis of Zebrin II ⁺/₋ stripes (red fluorochrome, white bars and text). Zonal boundaries for rat cerebellum were identified on the basis of Sugihara and Shinoda (2004) classifications. **A:** Highlighted fibre bridges zones AX and B (and potentially crosses into zone A) in lobule IV (scale bar: 200 μm ; region analyzed is "a" in panel D). **B:** In lobule V two highlighted SERT⁺ fibres bridge zone A and AX and one bridges zone AX and B (scale bar: 200 μm , region analyzed is "b" in panel D). **C:** In a more lateral region of Lobule VI, two highlighted SERT⁺ fibres cross zones X, AX and B and one bridges zone X and AX (scale bar: 150 μm ; region analyzed is "c" in Fig D). **D:** Zebrin II stripe pattern in cerebellar vermis, projected on a flattened map of cerebellar zonal architecture.

Fig 9. Summary: Organization of NA and 5H-T afferent fibres in the cerebellar cortex. Serotonergic fibres (5-HT) ascend to the molecular layer before bifurcating and projecting for relatively long distances in the medial-lateral plane, in a manner reminiscent of parallel fibres though with more rostral-caudal and dorsal-ventral deviation than is seen in parallel fibres (range indicated by crossed arrows*). Serotonergic fibres can certainly cross three longitudinal zones in the vermis and may cross more. Noradrenergic fibres (NA) project longer distances in the rostral-caudal plane than the medial-lateral plane, though the orientation preference is less complete than is seen in serotonergic fibres. The medial-lateral and dorsal-ventral deviations of noradrenergic

fibres are much smaller than their rostral-caudal extents (range indicated by crossed arrows †) but are larger than the equivalent rostral-caudal and dorsal-ventral deviations of the 5-HT fibres. The limited medial-lateral extents of the NA fibres could restrict their influence to a single zone or pair of zones if the fibre runs close to a zonal border. In many instances, the medial-lateral extent restriction could be sufficient to confine influence to a single microzone.

Highlights

- Serotonergic (5-HT) and noradrenergic (NA) afferent systems to the cerebellar cortex have unique and different geometries.
- 5-HT and NA fibre trajectories are strongly anisotropic and orthogonal to each other.
- Each 5-HT afferent runs along the long axis of its cortical folium crossing multiple zones and potentially signals to all.
- Each noradrenergic afferent runs parasagittally with small medial-lateral extent and may signal to one or few microzones.
- The geometry of these monoaminergic afferent systems suggests very different roles in cerebellar information processing.

