Review

The use of viral gene transfer in studies of brainstem noradrenergic and serotonergic neurons

S. Kasparov* and A. G. Teschemacher

Department of Physiology and Pharmacology, Bristol Heart Institute, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, UK

In contrast to some other neuronal populations, for example hippocampal or cortical pyramidal neurons, mechanisms of synaptic integration and transmitter release in central neurons that contain noradrenaline (NA) and serotonin (5HT) are not well understood. These cells, crucial for a wide range of autonomic and behavioural processes, have long un-myelinated axons with hundreds of varicosities where transmitters are synthesized and released. Both seem to signal mostly in ‘volume transmission’ mode. Very little is known about the rules that apply to this type of transmission in the brain and the factors that regulate the release of NA and 5HT. We discuss some of our published studies and more recent experiments in which viral vectors were used to investigate the physiology of these neuronal populations. We also focus on currently unresolved issues concerning the mechanism of volume transmission by NA and 5HT in the brain. We suggest that clarifying the role of astroglia in this process could be essential for our understanding of central noradrenergic and 5HT signalling.

Keywords: noradrenaline; serotonin; neurons; exocytosis; viral vectors; astrocytes

1. INTRODUCTION

Noradrenaline (NA) and serotonin (5-hydroxytryptamine, 5HT) are key neuromodulators in the mammalian brain. Over the last few decades, much effort has been dedicated to gaining information about the roles of these molecules in various brain functions and in different parts of the brain. The interest in these monoamines is justified because of the extensive range of human pathologies associated with malfunction of one or both of them. In the case of NA, these include depression, appetite disorders, chronic pain, sleep disorders, essential hypertension and some others (for references, see Kasparov & Teschemacher 2008). For 5HT, human data strongly implicate it in anxiety and depression, aggressive behaviour, control of appetite and also, more recently, in schizophrenia (Gonzalez-Maeso *et al.* 2008) and Parkinson’s disease (Caretti *et al.* 2008). In animal studies, 5HT has also been demonstrated to play a role in central control of pain (reviewed in Suzuki *et al.* 2004; Rice & Hill 2006; Hamel 2007) and sympathetic outflow (Howe 1985; Jordan 2005; reviewed in Coote 1990). Both monoamines are also implicated in various aspects of drug abuse. Thus, there is a substantial degree of overlap between them regarding human pathologies and the associated mechanisms. It is not surprising that antidepressant drugs which block re-uptake of NA, 5HT or both are used for treatment of essentially the

same groups of patients suffering from major depression and anxiety.

There are further interesting parallels. First, both NA and 5HT act via G-protein-coupled receptors (with the exception of the 5HT₃ receptor). These receptors activate relatively slow-signalling cascades compared with ionotropic receptors such as α -amino-3-hydroxyl-5-methyl-4-isoxazole propionic acid and *N*-methyl-D-aspartate or gamma-amino butyric acid A (GABA_A) receptors. Coupling to intracellular second messenger systems makes NA and 5HT better suited for lasting modulation of cellular activity than for rapid control of membrane potential and neuronal excitability. Second, both types of neurons have un-myelinated varicose axons. The varicosities represent specialized sites of transmitter release, but it is often forgotten that varicosities also represent obstacles for action potential propagation (Debanne 2004), preventing high fidelity of transmission in these systems. Third, both systems have cell bodies concentrated in a few clusters in the medullary–pontine part of the brain, but the axons of those relatively few neurons project extensively, covering essentially the whole central nervous system (CNS), from olfactory bulb to the spinal cord. Thus, a single axon with thousands of varicosities transverses numerous areas of the brain and evokes speculation as to whether release of NA or 5HT can occur in target-specific manner after activation of the brainstem monoaminergic nuclei.

Until recently, most of the research into central NA and 5HT transmission was focused almost entirely on the firing characteristics of these cells, distribution of their receptors in various parts of the CNS, actions

*Author for correspondence (Sergey.Kasparov@bristol.ac.uk).

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of the agonists and antagonists of NA or 5HT and the effects produced when either NA or 5HT nuclei were stimulated or inhibited in various ways. Little was known about the quantal characteristics of NA and 5HT release in mammalian CNS until it became possible to visualize living NA and 5HT neurons and their varicosities using expression of fluorescent proteins. This was achieved through viral vectors equipped with cell-specific promoters (CSPs), and has permitted an in-depth electrophysiological analysis of neurotransmitter release.

2. NEW INSIGHTS INTO THE QUANTAL CHARACTERISTICS OF NORADRENALINE RELEASE IN THE BRAIN

Despite the fundamental role of NA in brain function, the mechanisms regulating its release are still poorly understood. In fact, even the exocytotic nature of NA release was postulated but, until recently, never proved directly. Many important characteristics of central NA release, such as kinetics of quantal events and potential roles for multiple NA-containing vesicle populations, that could be anticipated based on previous electron microscopic (EM) studies remained undocumented. In contrast, the molecular processes underlying catecholamine (CA) exocytosis in peripheral cells, such as adrenal chromaffin cells, are well established. However, while neuroendocrine cells and central noradrenergic (NAergic) neurons may share many mechanistic features in respect to their transmitter release apparatus, the obvious dissimilarities in morphology, compartmentalization and vesicle populations between the two cell types suggest differences in the molecular composition of exocytotic machinery, vesicular matrix and intracellular signalling pathways (reviewed in [Teschemacher 2005](#)). Another unresolved issue was the distribution of release sites within NAergic neurons, a key determinant of the functional impact of secreted NA. Release from axonal varicosities will activate receptors distant from the neuron's cell body, for example in the spinal cord, hypothalamus or cortex. In contrast, somato-dendritic release could affect either the NAergic cells themselves in an autocrine fashion or adjacent cells in the same nucleus such as the nucleus tractus solitarius (NTS) which harbours the A2 NAergic group. Several lines of evidence suggested that NA release may occur in the proximity of NAergic somata ([Groves & Wilson 1980](#); [Egan *et al.* 1983](#)). To address the questions surrounding NA release in the brain, we developed an amperometric approach based on carbon-fibre microelectrodes. This made possible recordings of quantal release events from genetically targeted NAergic green fluorescent protein (EGFP)-expressing neurons in an organotypic brain-slice culture ([Teschemacher *et al.* 2005a,b](#)). The PRSx8 promoter that was used to drive EGFP expression, with few exceptions, operates specifically in NAergic and adrenergic neurons ([Hwang *et al.* 2001](#); [Lonergan *et al.* 2005](#)). EGFP expression in these slice cultures was consistent with the locations corresponding to A2 neurons in the nucleus of the solitary tract and the ventral A1 group ([Paxinos & Watson 1986](#)).

Carbon-fibre amperometry provides a quantitative measure of oxidizable transmitter per release quantum, and has been increasingly used over the past two decades, for example, to characterize release of NA in chromaffin cells in real time ([Travis & Wightman 1998](#); [Teschemacher & Seward 2000](#)).

Our study ([Chiti & Teschemacher 2007](#)) established several key characteristics of CA release from central neurons ([figure 1a,b](#)). Spontaneously occurring amperometric events, consistent with vesicle exocytosis, were found in A1 and A2 neurons in slice culture. These were present at cell bodies as well as axonal varicosities. We know from whole-cell patch clamp experiments in this preparation that the majority of NAergic neurons fire action potentials spontaneously ([Chiti & Teschemacher 2007](#); [Teschemacher *et al.* 2008](#)). Thus, amperometric events that appeared at a wide range of frequencies at different release sites could be related to the action potential activity of NAergic neurons, but also to the firing and signalling of neighbouring neurons, under these recording conditions. In line with this, a prominent reduction of axonal release events was seen in 1 μ M tetrodotoxin (TTX). However, a significant fraction of events persisted, indicating a role for action potential-independent release.

At both somata and varicosities, two populations of release events were observed ([figure 1b](#)). The predominant population (more than 95%) had a mean quantal content of approximately 1.2×10^5 NA molecules, which may originate from the 80–120 nm diameter dense core vesicles that have been found in central monoaminergic neurons by EM studies ([Bloom & Aghajanian 1968](#); [Nirenberg *et al.* 1995](#); [Pickel *et al.* 1996](#)). As intravesicular NA loading of vesicles is a dynamic process that depends on CA uptake and metabolism, a broad concentration range would be expected within actively signalling tissue. It is not known yet whether the small (40–60 nm) clear vesicles of monoaminergic neurons also contribute to this population of NA-release events. The minority of NA quanta (more than 3%) were more than an order of magnitude larger and released on average approximately 5×10^6 molecules of NA, which comes close to the chromaffin cell quantal size ([Albillos *et al.* 1997](#)). Because of their high NA content, such infrequent but large events released about one-third of total NA. If large complex (multi-peak) events are taken into account, their contribution is even larger ([Teschemacher *et al.* 2008](#); see below). There is scanty evidence for chromaffin-cell-like large dense-core vesicles in the rodent brain, which could explain why their exocytosis is a comparatively rare event. Future EM studies of central NAergic vesicle populations to investigate their contents will help to combine amperometric and morphometric evidence.

The presence of multiple types of secretory vesicles may have implications for the downstream effects of released transmitter in terms of the signalling range and cotransmitters present in different types of vesicles. It is tempting to speculate that NA released in large quanta might have better chances of diffusing further to reach additional cellular targets at an effective concentration within the brain tissue. Exocytosis

Figure 1. Amperometric characterization of NA and 5HT release from rat central neurons. (a) Examples of NA-release events recorded from different compartments (axonal varicosities and somato-dendritic regions) in medullary organotypic slices of rat. Note that the events shown on the right are large, which is reflected by the different scale bars. These large events are relatively rare but because of their high transmitter load, they may contribute in some cases over 80 per cent of the total CA release. (b) Analysis of quantal sizes of single events registered from NA neurons. Conversion of charges into cube roots (x -axis) was performed to accommodate for NA storage within spherical vesicles and revealed two major populations of quanta, the main population of frequent and small-sized events and rare large events. The 'main population' was well described by a Gaussian distribution. Large events (indicated by black arrow), clearly to the right of the main population, were registered in most recordings. Dotted bar, varicosities; filled bar, somata/dendrites. Adapted from Chiti & Teschemacher (2007). (c) Top—amperometric trace (raw data) demonstrating quantal release of 5HT registered from EGFP-fluorescent varicosities of rate raphe neurons. Below—examples of single events of various amplitudes. Note that many of these events are much smaller than what was found in NAergic neurons (a). (d) Analysis of quantal sizes of single events recorded from rat raphe 5HTergic neurons. Note that large events were registered only from somato-dendritic compartments of these cells. Dotted bar, varicosities; filled bar, somata/dendrites. Adapted from Benzekrouia *et al.* (2009a).

of different vesicle categories may also be differentially regulated (Bruns *et al.* 2000; Klyachko & Jackson 2002). We are only at the beginning of addressing the issue of the effective signalling range of NA fibres, the roles of various types of release events and the conditions under which NA-release events and signalling range may change. For example, when we treated slice cultures chronically with a known inhibitor of central NA reuptake, the tricyclic antidepressant amitriptyline, it had little effect on the frequency of release events, but significantly increased their quantal content, implicating a change in the releasable pool of NA (Chiti & Teschemacher 2007).

3. ALTERED CENTRAL NORADRENALIN SIGNALLING IN AN ANIMAL MODEL OF ESSENTIAL HYPERTENSION

Can reorganization of NA release also occur in pathological conditions? In a recent study, we have seen such a transformation in the C1/A1 catecholaminergic (CAergic; to include both adrenergic and NAergic) cell group in the rostral ventrolateral medulla (RVLM; Teschemacher *et al.* 2008). Essential hypertension, a major medico-social problem of the recent time, is characteristically accompanied by increased central sympathetic drive as found in both human patients and the spontaneously hypertensive rat (SHR), an established animal model of this disease (Iriuchijima *et al.* 1975; Judy & Farrell 1979; Grassi 2004; Schlaich *et al.* 2004). The link between brainstem CAergic activity, sympathetic output and blood pressure was made more than 30 years ago and is documented extensively (reviewed in Kasparov & Teschemacher 2008). Yet, it had not been possible to clearly identify the differences in the signalling properties of these neurons between hypertensive and normotensive phenotypes because suitable methods were lacking. Using viral gene targeting of CAergic neurons, it has been possible to test for three hypothetical differences between CAergic transmission in the normotensive Wistar rat (WR) and SHR (Teschemacher *et al.* 2008).

First, based on our previous observations described earlier, we investigated whether CA in SHR may be stored and released differently. This hypothesis was supported by previously published evidence of morphological changes in storage vesicles in SHR and their increased ability to take-up ^3H -NA (Rho *et al.* 1983), higher expression of norepinephrine transporter in SHR brainstem cultures (Veerasingham *et al.* 2005) and increased tyrosine hydroxylase expression in the SHR ventrolateral medulla (Reja *et al.* 2002). We therefore reasoned that the structural differences in CA-storing vesicles and/or NA synthesis and transport could translate into enhanced packaging and release of CA in SHR. In other words, it could be that in SHR, CAergic volume transmission is amplified, increasing its effect on various cellular targets in areas critical for control of sympathetic outflow. Our study provided evidence in support of this first hypothesis (Teschemacher *et al.* 2008). It appears that in SHR C1 varicosities release almost double the amount of CA via large quanta (transmitter loads comparable to

those found in peripheral chromaffin cells) when compared with WR (figure 2). In contrast, in A2 neurons, we found no significant difference in quantal distribution between WR and SHR. We speculate that such reorganization of the CA vesicular pools may have a major impact on the range of downstream signalling by C1 varicosities (figure 2). The RVLM provides tonic stimulatory input to many CNS sites of autonomic control, but most notably the sympathetic pre-ganglionic neurons (Chalmers *et al.* 1981; Dampney *et al.* 1982; Reis *et al.* 1984). Consistent with the 'volume' mode of transmission, varicosities of C1 neurons in the spinal cord seldom form tight synapse-like appositions with pre-ganglionic sympathetic neurons (Anderson *et al.* 1989). As large release events are very fast in the brain (rise times less than 3 ms, duration less than 10 ms in most cases; Chiti & Teschemacher 2007), they deliver copious quantities of CA into the extracellular space essentially instantaneously. Thus, CA released in volume-mode transmission might spread further from the release site than when released in small quanta. When released within synaptic appositions (Morrison *et al.* 1988; Anderson *et al.* 1989), a high quantal load may result in synaptic spillover and add to volume transmission. Hence, by releasing bulk loads of transmitter, C1 varicosities could signal to a wider range of cellular targets in the SHR. In addition, by analogy with chromaffin cells or peripheral sympathetic neurons, large vesicles are likely to release copious amounts of cotransmitters (such as ATP, chromogranin, neuropeptide Y), which could play an additional important signalling role (Winkler & Westhead 1980; Phillips 1982; Fillenz 1990).

Second, previous work had suggested that central CAergic neurons in the SHR could be electrically 'hyperactive' and generate action potentials at higher rates than in normotensive animals. Higher discharge rates had been previously reported in neurons (some of which could be CAergic) of the RVLM in neonatal SHR (Matsuura *et al.* 2002). However, when we tested CAergic cells directly, we were unable to confirm this hypothesis because A2 and C1 neurons in SHR were not intrinsically more excitable than in WR (Teschemacher *et al.* 2008).

The third hypothetical difference was that the excitatory effect of angiotensin II (AngII) on these neurons, or the characteristic AngII-induced Ca^{2+} mobilization (Teschemacher & Seward 2000), may be enhanced in the SHR. Central AngII has been implicated in pathogenesis of excessive sympathetic activity and hypertension (at least in animal models) by numerous studies (Phillips *et al.* 1977; Faber & Brody 1984; Itaya *et al.* 1986; Wu & Berecek 1993) and it was, therefore, conceivable that stronger activation of CAergic neurons by central AngII could lead to a higher transmitter release in SHR. AngII, indeed, depolarized many A2 and C1 neurons and triggered $[\text{Ca}^{2+}]_i$ elevations, presumably from the intracellular stores. Unexpectedly, SHR A2 neurons were less sensitive to this critical neuromodulator, both in terms of depolarization and Ca^{2+} mobilization, while an opposite trend was evident in C1 cells (Teschemacher *et al.* 2008). Taken together, these observations confirm that the changes associated

Figure 2. Amperometric analysis of transmitter release from varicosities of C1 and A2 neurons. Relative contributions of small (top half) and large (lower half), single (plain) and complex (dotted) release events to total CA release in WR and SHR. Note that the majority of CA release in C1 varicosities of SHR occurs in large quanta while the contribution of small events in C1 varicosities is reduced. Release from large quanta in SHR was increased, and of small quanta decreased, not only in relative, but also in absolute terms. These differences are area specific and not evident in A2 varicosities. Plain bar, small single-spike events; dotted bar, small complex events; filled bar, large single-spike events; large dotted bar, large complex events. Adapted from Teschemacher *et al.* (2008).

with the hypertensive phenotype are not universal across all the central neurons equipped to synthesize CA. Instead, the differences revealed in C1 are in some respects opposite to those in the A2 area.

The finding that A2 neurons in WR responded to AngII more vigorously than in SHR raises the possibility of a role of A2 neurons as part of an anti-hypertensive homeostatic mechanism that may be compromised in SHR (Snyder *et al.* 1978; Talman *et al.* 1980b; reviewed in Kasparov & Teschemacher 2008). More than three decades ago, it became apparent that A2 neurons may be important for blood pressure regulation and could be a key link in the mechanisms of action of central anti-hypertensive drugs such as clonidine and α -methyl-noradrenaline. Destruction of A2 neurons, using the neurotoxin 6-hydroxydopamine (6-OHDA), destabilised arterial pressure by causing lability (Talman *et al.* 1980c), without affecting mean arterial pressure (Talman *et al.* 1980a). However, whether A2 neurons are involved in mediating or modulating the baroreflex or play a role in long-term control of blood pressure remained uncertain. The problem is further confounded by the fact that 6-OHDA is not selective and also, for example, destroys 5HT-containing neurons projecting to the NTS (Itoh *et al.* 1992).

The function of A2 in blood pressure control *in vivo* was addressed in another study (Duale *et al.* 2007). Our approach was to avoid some of the previous limitations and electrically 'silence', rather than destroy, A2 neurons. We used viral vectors with PRS $\times$ 8 promoter to express in A2 neurons a human inwardly rectifying potassium channel (hKir2.1; Johns *et al.* 1999). To avoid the retrograde transduction of NAergic neurons that occurs when using adenoviral vectors (AVVs), (Loneragan *et al.* 2005), we

employed vesicular stomatitis virus (VSV) G protein-pseudotyped lentiviral vectors (LV). The hyperpolarizing effect of Kir2.1 expression was confirmed in that study in experiments on PC12 cells, and more recently, this has been also verified in CA neurons in organotypic slice cultures (P. W. Howorth, A. G. Teschemacher & A. E. Pickering 2009, personal communication).

Using this approach, we have been able to assess the role of A2 neurons chronically by reducing their electrical excitability while monitoring cardiovascular parameters and body fluid homeostasis in freely moving normotensive rats and SHR via radiotelemetry. In both rat strains, WR and SHR, virally mediated expression of hKir2.1 in A2 neurons led to a chronic and sustained elevation of arterial pressure with an earlier onset of the effect in SHR. In addition, lability of arterial pressure increased in both rat strains, although there was no change in either heart rate or cardiac baroreceptor reflex gain. While the effect on blood pressure was more evident in SHR, we could not reveal any measurable difference in either spread of transgene or basic membrane properties of A2 neurons between the two rat strains. However, our analysis indicates that the autonomic mechanisms underpinning the hypertension and blood pressure lability may differ between WR and SHR. We found a significant increase in the very low-frequency component of the power spectrum of the systolic blood pressure in both rat strains after hKir2.1 transduction, but only in SHR did we observe an elevated low-frequency component. As these changes occurred in parallel with the rise in arterial pressure, they might provide clues to the plausible mechanisms of this phenomenon. It is possible that the increase in the low-frequency component in the SHR reflects an increase in sympathetic activity (Waki *et al.* 2006). Interestingly, this effect was absent in the normotensive WR, perhaps indicating the hyperexcitable state of the sympathetic nervous system in the SHR. We also noticed that expression of hKir2.1 in WR but, surprisingly, not in SHR, resulted in a significant decrease in water intake and urine output during the first 7 days post-viral transduction. This suggests that A2 neurons have qualitatively different homeostatic functions in these two rat strains.

Previous chemical lesioning studies using 6-OHDA to destroy A2 neurons reported lability in arterial blood pressure but failed to find hypertension. This may relate to the methods adopted. Indeed, destruction of NAergic neurons using 6-OHDA is likely to cause plastic changes in nearby neuronal architecture and rewiring with an unpredictable outcome. Interestingly, a transient rise in arterial pressure was evoked 3 h after 6-OHDA treatment, but by 24 h arterial pressure was back to control (Talman *et al.* 1980a), supporting the notion of neuronal compensation.

It is of interest that intermittent hypoxia, a known stimulant for hypertension and sympatho-excitation, is associated with a reduction in the tyrosine hydroxylase content and its phosphorylation in brainstem CAergic neurons, including those in the A2 region (Gozal *et al.* 2005). This might be expected to cause a reduction in CA release. In this regard, our attempts to depress the electrical excitability of A2 neurons might, in part, mimic the effect of intermittent hypoxia and hence contribute to the hypertension (Gozal *et al.* 2005).

Figure 3. (a) Principle of TSTA amplification. In order to increase the activity of a relatively weak CSP, this system employs a chimeric transcriptional enhancer, GAL4|p65. p65 is a strong mammalian transactivator, but in this construct, its DNA-binding domain has been substituted by the GAL4 from yeast. The GAL4-binding domain does not bind to mammalian sequences and therefore GAL4|p65 specifically potentiates expression of the target expression cassette. Several implementations have recently been published (Liu *et al.* 2006, 2008a,b). (b) Application of this strategy to visualize 5HTergic neurons. AVVs were constructed based on a 3.6 kb fragment of rat TPH-2 promoter and the strategy depicted in a. (i) EGFP-fluorescent neurons and (ii) varicosities could be directly visualized and studied in organotypic slice cultures of the rat (Benzekroufa *et al.* 2009a).

In summary, using viral vectors for targeted gene expression in central CAergic neurons, we have obtained the first information about the quantal characteristics of NA release in mammalian brain. It was found that NA release occurs in quanta of different sizes and that chronic treatment with an uptake blocker shifts the balance in favour of large quanta. Moreover, in SHR, a similar shift in release mode occurs in one CAergic cell group (C1/A1), which has been implicated in control of sympathetic activity by many previous studies. It turns out that the changes in CAergic signalling in SHR are not universal because in A2 cells we detected essentially an opposite trend, consistent with the notion that the A2 neurons are a component of a central 'anti-hypertensive' circuit. In support of this point, genetic 'silencing' of A2 neurons using over-expression of Kir2.1 resulted in sustained elevation of blood pressure.

4. VIRAL VECTORS FOR SELECTIVE TARGETING OF SEROTONERGIC (5HT_{ERGIC}) NEURONS

As mentioned earlier, 5HT_{ERGIC} and NAergic neurons are in some respects similar and it would be interesting to compare their features. Direct characterization of NA release in mammalian brain was made possible using a viral vector with PRSx8 promoter that labels mainly CAergic cells (Lonergan *et al.* 2005). We therefore decided to develop a viral system for targeting 5HT_{ERGIC} neurons.

Two problems had to be overcome for that project to succeed. First, it was necessary to find a promoter that would be sufficiently selective but also adequately short in order to be packaged into a conventional vector, such as an AVV or LVV. The most obvious targets were the sequences located at the 5' end of the recently

discovered tryptophan hydroxylase-2 (TPH-2) gene, the key enzyme in central neuronal 5HT synthesis (Walther *et al.* 2003). The second requirement was the potency of expression, because in order to be useful, the promoter had to be able to drive visible expression of a fluorescent marker, such as EGFP. This not only allows visualization of living cells but also ensures that any functional gene expressed in these cells will actually change their physiology.

Mammalian CSPs are usually much less active than those derived from viral pathogens. In order to enhance the activity of a CSP without the loss of specificity, we used a two-step transcriptional amplification (TSTA) strategy (Nettelbeck *et al.* 1998; Iyer *et al.* 2001; Liu *et al.* 2006). TSTA uses artificial transcriptional enhancers to potentiate expression. Such enhancers bind to sequences absent from mammalian genomes that need to be placed upstream of a weak CSP (figure 3). Previously (Liu *et al.* 2006, 2008a,b), we successfully used a potent recombinant transcriptional activator consisting of a GAL4-binding domain fused to a part of the transcriptional activation domain of NF-kBp65, one of the strongest mammalian transcriptional activators known. We therefore identified a sufficiently specific targeting sequence in the promoter region of the TPH-2 gene, and amplified its expression efficiency using TSTA (figure 3b). Viral vectors based on TPH-2 promoter fragments have specificity in excess of 95 per cent based on double staining for TPH-2 (Benzekroufa *et al.* 2009a,b).

Using these vectors, we have performed initial characterization of the quantal 5HT release from rat midline raphe neurons in slice culture (figure 1c,d). Our micro-amperometry recordings revealed that 5HT, just like central NA (Chiti & Teschemacher 2007), is released by exocytosis from cell bodies as

Figure 4. Tight interpositions between NAergic neurons and astrocytes. In an organotypic slice culture preparation, EGFP was expressed in NAergic neurons using PRSx8-EGFP AVV. An AVV hCMV-DsRed was co-applied. In accordance with our previously published observations, hCMV is inactive in NAergic neurons and under these experimental conditions expresses almost exclusively in astroglia. Multiple interpositions between NAergic processes and astrocytes can be seen (for example, *a*, yellow arrow). In some cases, the varicose NAergic axons loop around astrocytic processes (*a*, blue arrow). In many cases, NAergic processes can be found embedded within the somata of astrocytes (*b*, yellow and blue arrows; *c*, yellow arrows). Not infrequently, astrocytic processes pass through the dendrites of NAergic neurons (*d*, yellow arrow). Please note that only a small fraction of the astrocytes present within the tissue was made fluorescent in these experiments and is visible on the images by using moderate quantities of the corresponding vector.

well as varicosities. Quantal sizes detected were markedly smaller than those of NA but quanta registered at somato-dendritic locations were approximately 20 per cent larger than those registered from axonal varicosities. Interestingly, as for NA, we found some exceptionally large release events discharging on average approximately 800 000 molecules of 5HT (Benzekhrouta *et al.* 2009a). The likeliest anatomical substrate for the main population of 5HT-release events are dense core vesicles, identified in central monoaminergic neurons using EM (Bloom & Aghajanian 1968; Beaudet & Descarries 1981). For the large 5HT-release events, a morphological correlate remains to be found. Again, it is possible that release in larger quanta could be an important mechanism regulating the efficacy of 5HT signalling to downstream targets.

5. WHAT DETERMINES THE QUANTITY OF MONOAMINES DELIVERED BY NORADRENALINE AND SEROTONIN NEURONS TO DIFFERENT AREAS OF THE BRAIN?

Now that measurements of NA and 5HT release in the CNS are possible and that patch clamping combined

with $[Ca^{2+}]_i$ measurements can be performed also, we should be able to tackle this long-standing question. As monoamines are released from cell bodies and axonal varicosities, it relates not only to the remote parts of the CNS, which are in receipt of NAergic or 5HTergic innervation, but also the nuclei that harbour the somata (for example, NTS for A2 NAergic neurons or raphe nuclei for 5HTergic neurons). As mentioned earlier, we found that NA varicosities in organotypic slice cultures still release NA in the presence of TTX, although at a considerably slower frequency (Chiti & Teschemacher 2007). In acute slices from the locus coeruleus (Huang *et al.* 2007), it was found that only approximately 25 per cent of NA release from soma evoked by membrane depolarizations was blocked by TTX. Nevertheless, several studies demonstrate that release of monoamines in the target areas is action-potential dependent, although the data concerning this issue remain controversial and in some cases, rather surprising. For example, when locus coeruleus was stimulated electrically, stimulation at 10 and 20 Hz was unable to trigger NA release in the spinal cord as detected using cyclic voltammetry, and it required several seconds of

stimulation at 50–100 Hz to obtain a clear response (Hentall *et al.* 2003). This is in stark contrast with the fact that locus coeruleus neurons usually generate action potentials at frequencies of less than 5 Hz and can generate bursts of up to 50 Hz only for brief periods. Moreover, the average latency of these responses in the spinal cord was 4.5 s, much longer than can be explained by action potential propagation. Similar observations were later published by the same group in relation to the 5HT release in the dorsal horn evoked by stimulation of the nucleus raphe magnus (Hentall *et al.* 2006). Berridge & Abercrombie (1999) used microdialysis to study the relationship between NA efflux in the pre-frontal cortex and the multi-unit activity of the locus coeruleus (LC). That study found that NA outflow was strongly enhanced (approx. 300%) when LC neurons were activated with betanecol, which apparently increased their discharge rate only to 200 per cent of the basal rate. At the same time, stronger stimulation that resulted in firing rates of greater than 500 per cent led to almost no additional increase in NA efflux. This suggests that only moderate stimulation frequencies should be required to trigger maximal NA output, which is at odds with the studies cited above (Hentall *et al.* 2003, 2006). Moreover, somatic NA release could not be evoked in LC neurons at frequencies less than 20 Hz, while the delay between membrane depolarization triggered via a patch pipette and amperometric spikes caused by NA release from somata was more than 100 ms (Huang *et al.* 2007). These observations highlight the fact that in monoaminergic neurons, the relationship between action potential frequency and the signalling to downstream targets is nonlinear and not well understood.

6. WHO IS OUT THERE TO LISTEN?

What are the downstream targets of transmitters released from NAergic or 5HTergic varicosities in their projection areas? Analysis of EM studies published mainly in the previous two decades reveals a remarkable fact. For example, Seguela *et al.* (1990) concludes that

In contrast to their unlabeled counterparts, these [putative NAergic—SK] profiles rarely showed a membrane differentiation characteristic of a synaptic contact (junctional complex). As extrapolated for whole varicosities after linear transformation (double reciprocal plot), this proportion was 17% or 26% depending on the stringency of the criteria used in identifying the junctional complex. The same analysis provided a figure of 98% for the control population. (Seguela *et al.* 1990)

In other words, in the cortex, only approximately one-fifth of NA-release sites actually form any synapse-like structures. In case of 5HT, some authors reported more incidences of synapse-like specializations on putative 5HT varicosities, but overall, as reviewed in Soghomonian *et al.* (1998), a paucity of synaptic contacts established by 5HT varicosities appears to be the rule, rather than exception. Of note, attempts to reveal

‘post-synaptic’ responses attributable to release of 5HT or NA in neurons of various target areas have not yielded a convincing case for the existence of such responses, even in the case of 5HT₃-receptor-mediated signalling, in spite of the fact that these receptors are the only known ionotropic variety within the 5HT- or NA-receptor families. For example, trains of electrical stimuli (at approx. 200 Hz) were able to evoke depolarizing potentials sensitive to 5HT-receptor blockers in a small subset of neurons in amygdala (Sugita *et al.* 1992). There, potentials were fairly slow, lasted approximately 40–60 ms and reversed around 0 mV, which, given the ion concentrations used in that study, suggested a non-selective cation conductance. In a fraction of cortical interneurons, Ferezou *et al.* (2002) found that single-shock field electrical stimulation evoked non-GABA-mediated inward post-synaptic currents, which could be blocked by a 5HT₃-receptor antagonist. However, these currents were fast (less than 3 ms) and large (mean amplitude –240 pA). They were followed by a much smaller and slower outward 5HT₃ antagonist-sensitive current that lasted 25–40 ms. The dynamic of this slow current was much closer to that in Sugita *et al.* (1992), but this was almost certainly a K⁺ conductance, which may not lead to neuronal activation typically expected of 5HT₃-receptor activation when triggered by bath-applied ligands. Thus, in these two studies, 5HT₃-mediated currents were rare and their key electrophysiological characteristics appear to be dissimilar. In neurons of the hypoglossal motor nucleus, some slow (35–50 s) post-synaptic responses sensitive to 5HT₂-receptor blockers were reported (Bobker 1994). In the case of NAergic transmission, local electrical stimulation evoked post-synaptic potentials in raphe neurons that were sensitive to the α_1 -receptor antagonist prazosin. These potentials appeared at a latency of 250 ms and lasted almost a minute (Oleskevich & Williams 1995). Similar long-lasting NA-dependent components of post-synaptic responses were isolated in cortical neurons after repetitive field stimulation (Benardo 1993). Such slow events are difficult to reconcile with the ‘synaptic’ mode of signalling. Thus, the evidence for reasonably fast, synaptically mediated responses in target neurons for both 5HT and NA is limited. This cannot be explained by the fact that almost all the receptors for these monoamines are G-protein coupled. It is known that GABA_B receptors mediate bona fide inhibitory post-synaptic currents that have latencies in the range of tens of milliseconds and last a few hundreds of milliseconds (Kaila *et al.* 1993). The key difference is that GABAergic axons release their transmitter into genuine synapses where the ‘post-synaptic cell’ is the target neuron while the majority of NA- and 5HT-release sites do not directly face any neuronal post-synaptic membrane. However, monoamines released from varicosities do not diffuse to target neurons through empty extracellular space. There is no such space in the brain and, invariably, all EM studies demonstrate extensive interpositions of NA-/5HT-release sites and astroglia. Associations of NAergic varicosities with astroglia can be visualized

by inducing these cell types to express different coloured fluorescent proteins such as EGFP and DsRed (figure 4). In many cases, varicosities wrap around astrocytes or lie in 'pockets' created by cell bodies and processes of astroglia. The hypothesis of glial involvement in NA signalling is not new and the data supporting it is extensive (reviewed in Stone & Ariano 1989; Hertz *et al.* 2004). We speculate that it is possible that a significant proportion of NA, especially released in 'small quanta' mode, actually is destined for astrocytes, rather than neurons. There is a variety of ways in which astrocytes can then affect the activity of the adjacent neurons. For example, astrocytes have been demonstrated to release homocysteic acid upon stimulation of β -adrenergic receptors (Do *et al.* 1997) and homocysteic acid is a known agonist of glutamate receptors (Knopfel *et al.* 1987; Ito *et al.* 1991). Activation of adrenergic receptors also activates glycogen breakdown in astrocytes, and there are many indications that activated astrocytes can then affect the function of adjacent neurons via some metabolic pathway or by conversion of newly generated pyruvate into glutamate which glia can release (reviewed in Hertz *et al.* 2004). Possibly, an even more important pathway is via the effects on the growth factor brain-derived neurotrophic factor released by astroglia, which could be mediated via both α - and β -adrenoceptors (Juric *et al.* 2008). According to the estimates made by Morin *et al.* (1997), the density of β -adrenoreceptors is higher on astrocytes than on neurons isolated from the cortex and cerebellum. Interestingly, recent studies in a glioma C6 cell line suggest that 5HT may activate glial neurotrophic factor production (Hisaoaka *et al.* 2007; Tsuchioka *et al.* 2008). Therefore, there is an intriguing possibility that at least some of the effects of NA or 5HT signalling in the brain involve astrocytes as intermediates (Stone & Ariano 1989).

Fortunately, there are now viral vectors that selectively target all three of the cellular phenotypes in question, NAergic and 5HTergic neurons and astrocytes. The introduction of opto-genetic experimentation on the brain whereby light-sensitive ion channels are expressed in target cells (Nagel *et al.* 2003, 2005; Zhang *et al.* 2006) opens a new window of opportunity to gain a better understanding of monoaminergic transmission in the brain. Optical excitation of NAergic and 5HTergic fibres in target areas should help to find out more about the mechanisms of transduction in these systems. The most pertinent questions as we see them are the following: (i) What determines the release of NA and 5HT in axonal target areas? (ii) Can release in differently sized quanta actually trigger different physiological responses? (iii) Which diffusion processes describe the spread of NA and 5HT within brain tissue? (iv) What is the role of astroglia in communication between monoaminergic neurons and other neuronal populations? Viral vectors and other novel genetic tools will be indispensable for addressing these questions.

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