Microelectrode investigation of neuroneal ageing from a single identified neurone

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Microelectrode amperometry is uniquely suited for characterising the dynamics of neurotransmitter release, as it offers unparalleled spatial and temporal resolution. We have used carbon fibre microelectrodes to study release of the monoamine neurotransmitter serotonin (5-HT) and the gaseous transmitter nitric oxide (NO) in intact central nervous system of the water snail, *Lymnaea stagnalis*. Analysis of spontaneous vesicular release of 5-HT and depolarisation-induced release of NO reveals significant differences with ageing that may be associated with changes in protein structure and function.

Introduction

Neurotransmitters are key signalling molecules responsible for propagating signals between neurones and from neurones to muscles. Individual neurones typically contain many signalling molecules which act together to shape the response of the target cell. Common combinations include a classical transmitter co-localised with one or more peptides and gaseous signalling molecules.[1,2] Changes in the release pattern of any of these signalling molecules could affect cell–cell signalling and have a dramatic effect on the behavioural output of the circuit.[3,4] Since the monoamines and the gaseous transmitters such as nitric oxide (NO) are electrochemically active their release can be monitored in physiologically realistic preparations using microelectrodes.

Microelectrodes have been widely used to study single vesicular release of neurotransmitters and also to study NO release. Since Ralph Adams' pioneering work[5] microelectrode measurements have provided a powerful tool to obtain dynamic information about the release of neurotransmitters both *in vivo* and *in vitro*. The monoamines are principally released following vesicular fusion with the cell membrane in discrete events, typically lasting a few microseconds. There are currently no alternatives to microelectrode methods for the selective direct monitoring of this fundamental neurochemical process. Ewing and Amatore have widely studied the regulation of vesicular release using isolated adrenal chromaffin cells or PC12 cells,[6–10] both of which are convenient surrogates for neurones, since they are larger than mammalian neurones and the vesicles contain much higher concentrations of neurotransmitter molecules, making release events easier to detect. Due to the dynamic nature of the transmission process, the vesicular event obtained from amperometry can be utilized to understand alterations in the release and clearance of the neurotransmitter investigated.

Changing demographics in the developed world have led to great interest in the biology of ageing. We are interested in the neurological changes in normal ageing of the brain which, in contrast to neurodegenerative diseases are believed to be due to changes in the strength of neuroneal connections.[11] These changes could be due to pre-synaptic phenomena such as changes in synthesis or release of transmitters or post-synaptic such as changes in receptor dynamics, expression or sensitivity. Whilst there have been reports on the effect of ageing on vesicular release in isolated PC12 cells,[12] there have been no reports to date on the dynamics of transmitter release in intact central nervous system (CNS). We have chosen to study the effects of age on transmitter release from a well characterised neurone, the cerebral giant cell (CGC) of the pond snail, *Lymnaea stagnalis*. This neurone has previously been shown to contain serotonin and its effects on target cells can be blocked by the addition of serotonergic antagonist.[13] The effects of serotonin on target neurones have also been shown to be altered with increasing age.[14] Additionally, the cell contains the enzyme nitric oxide synthase (NOS) giving it the potential to release the gaseous neurotransmitter NO.[15,16]

We have previously reported on correlates between behaviour and cumulative transmitter levels assessed by HPLC.[17,18] Here we report the use of microelectrodes in the amperometric mode to monitor the dynamics of the release of two different signalling molecules, *viz*. a vesicularly released monamine (serotonin, 5-HT) and a gaseous transmitter (nitric oxide, NO), in intact neuroneal systems.

Experimental

Microelectrode fabrication

Basic carbon fibre electrodes were prepared using a method adapted from Millar and Pelling[19] with subsequent modification to prepare microdiscs, rather than micro-cylinders. In brief, 7 and 30 μm carbon fibres were sonicated in acetone for up to 30 min and then cleaned in methanol and de-ionised water and

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dried using nitrogen. Glass capillaries (0.69 mm I.D., Clark Electromedical Instruments, Reading, UK) were pulled using a pipette puller (Narishige, Tokyo, Japan) and the tips were polished down to facilitate the insertion of the carbon fibre. The capillaries were then rinsed with ethanol and de-ionised water and dried with nitrogen. The carbon fibre was sealed in the capillary using de-gassed CY1301 epoxy resin and HY1300 hardener mixed in a 2.6 : 1 (v/v) ratio (Robnor resins Ltd, Swindon, Wilts, UK) and dried and cured for 72 h at room temperature. Connection was achieved by contacting a 0.25 mm silver wire using silver epoxy (RS components). A 1 mm gold pin was soldered to the silver wire flush with the glass capillary. The exposed tip of the carbon fibre was cut to an approximate length of 3 mm and cleaned in de-ionised water and dried under nitrogen. The shaft of the fibre was then insulated using Lectraseal anodic paint (LVH Coatings Ltd, Birmingham). Using a micro-manipulator the carbon fibre was located centrally in a platinum coil, which had a diameter of 2 mm, and was immersed in the anodic paint (dilution of 10 : 1 anodic paint : de-ionised water). To coat the carbon fibre a voltage of 2 V was applied for 1 min with the cathode connected to the platinum coil and the carbon fibre as the anode. The anodic paint was then cured for 20 min by placing the carbon fibre in the oven at 160 °C. This process was repeated four more times and the voltage was increased (3 V, 4 V, 6 V and 8 V) for each subsequent coating. The carbon fibre was then cut using a scalpel to expose a carbon disk electrode.

NO sensor fabrication

For detection of NO, 30 μm carbon fibre microelectrodes (prepared as above) were coated with multiple selective polymeric film members. Electrodes were initially coated with a Nafion film layer. The electrode was dip coated 3 times in a 5% v/v Nafion in ethanol solution and after each coating was left to air dry. After the third coating the electrode was heat cured at 200 °C for 4 min. The electrode was then placed in 0.1 M sodium hydroxide containing 10 mM eugenol (Fluka). The electrode was cycled at potentials between 0 and 1.25 V at a scan rate of 1 V s^{-1} for 25 cycles in order to electropolymerise the eugenol to the electrode surface.[20]

Experimental animals

All animals were bred in house at the University of Brighton. Animals were kept in large tanks at 18–20 °C on a 12 h light/dark cycle in copper-free tap water. They were fed on alternate days with either lettuce or fish flakes (Tetra UK Ltd.). Animals were kept in groups of up to 600 in large circulating tanks at a stocking density of approximately 1 snail per litre. Two age groups were examined, 3–4 months (young) and 11–12 months (old). 3–4 months was chosen for the young animals as it represented the earliest age when the animals were sexually mature. 11–12 months was chosen to represent old age as this was the time point when approximately 20% of the population survived.[21]

Isolated CNS preparation

The main ganglionic ring including the cerebral ganglia and a pair of buccal ganglia with a small piece of the pro-oesophagus still attached was removed from *Lymnaea* and then mounted in a Sylgard (Corning, UK) lined chamber that was continuously perfused with *N*-[2-hydroxyethyl]piperazine-*N*′[ethanesulfonic acid] HEPES-buffered saline (concentration in mM: NaCl 50; KCl 1.7; $CaCl_2$ 4; $MgCl_2$ 2; HEPES 10; pH 7.8) at a rate of approximately 1 ml min^{-1}. The right cerebral ganglion was de-sheathed using fine forceps to allow recordings from the CGC. The remaining connective tissue sheath covering the ganglia was enzymatically treated with 0.1% protease (Sigma Type XIV; Sigma UK) prior to commencing experiments.

Detection of 5-HT and NO from single neurones

Age-related measurements were carried out from young (3–4 months) and old (11–12 months) snails. For electrochemical measurements of 5-HT and NO, the electrode was placed on the cell body of the CGC neurone. All measurements were carried out using an Axoclamp 200B amplifier (Axon Instruments, CA, USA).

5-HT release was measured using an uncoated 7 μm carbon fibre electrode held at +750 mV *vs.* Ag|AgCl reference electrode. Two diameters of fibre were compared for their ability to detect serotonin release events; 7 μm and 30 μm. The amperometric signals were sampled at 25 kHz and filtered at 5 kHz using a 4 pole Bessel filter prior to digitization using a Micro 1401 A/D converter (CED, UK). The effect of the selective serotonin re-uptake inhibitor (SSRI) fluoxetine (Sigma-Aldrich, UK) on the release of 5-HT was investigated. Fluoxetine was applied to the soma of the CGC by means of a local superfusion pipette placed within 100 μm of the cell. Cells were continually perfused with normal HEPES-buffered saline and switched to a solution containing 10 nM fluoxetine in HEPES-buffered saline for periods of 10 min, before reverting back to perfusion with normal HEPES-buffered saline. Flow rates from the superperfusion pipette were 0.2 ml min^{-1} and the pipette had a dead space of ≈50 μl thus minimizing mixing of the solutions.

For detection of NO, a Nafion/poly-eugenol coated 30 μm disc electrode was held at +850 mV *vs.* Ag|AgCl reference electrode. The data were sampled at 100 Hz and digitised using a Micro 1401 A/D converter (CED, Cambridge, UK). Data storage and analysis was carried out using Spike 2 software (CED, Cambridge, UK). NO release was evoked by superfusing the cell with a high Ca^{2+}/high K^+ HEPES buffered saline (concentration in mM: NaCl 12.2, KCl 30, $CaCl_2$ 10, $MgCl_2$ 2.0, HEPES 10.0, pH 7.9). The superperfusion pipette containing the high Ca^{2+}/high K^+ buffer was placed within 100 μm of the cell body and cells were perfused with the saline for 100 s at a rate of 0.2 ml min^{-1}. The ability of L-NAME to inhibit high Ca^{2+}/high K^+ evoked responses was examined by perfusing cells for 100 s with a solution of 5 mM L-NAME made in high Ca^{2+}/high K^+ buffer following the control stimulus. Blank responses were obtained by placing the electrode well away from the CNS and perfusing with the high Ca^{2+}/high K^+ ringer, HEPES buffered saline and 5 mM L-NAME in high Ca^{2+}/high K^+ ringer to determine if any of the solutions were electroactive or otherwise evoked a current. Control measurements were also conducted from neurones

that were known from previous studies not to contain the NOS enzyme.[15,16]

Results and discussion

Detection of vesicular serotonin (5-HT) release

Spontaneous vesicular release of 5-HT was recorded from the cell body of an identified serotonergic neurone, the CGC. Fig. 1A shows a typical trace of spontaneous 5-HT release events from a young snail, where sample individual events are shown on an expanded time base. The events were a combination of single and multiple vesicular events, therefore selection criteria were required to ensure that only single vesicular events were identified for subsequent analysis. The first derivatives of smoothed base-line corrected recordings for each event enabled discrimination between "simple" events and "multiple" events based on the zero crossings. Fig. 1B shows examples of "simple" and "multiple" events with associated first derivatives of the current shown underneath each event. The line above the differential is 4.5 times rms of the noise. "Simple" events consisted of a single rising and falling phase, while "multiple" events consisted of multiple rising and falling phases.[22] Only simple events were analyzed in this paper.

From these events the time constant of decay (τ) from the decay phase of the signal and the charge (Q) were measured as shown in Fig. 1C. Q was calculated from the area of the vesicular event and therefore can be related to the number of molecules per vesicle based on Faraday's law. This has been presented in other studies of single release event from PC12 and chromaffin cells.[6–10] The falling phase of the individual

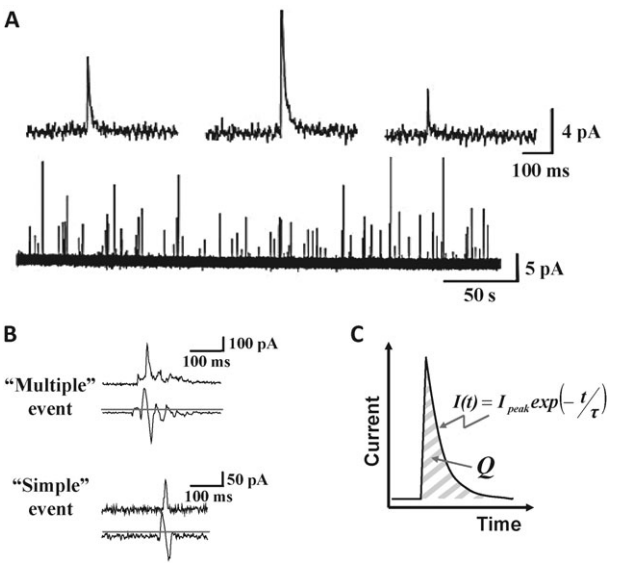

Fig. 1 Characterization of release events. In (A) a sample trace is shown, showing spontaneous release of 5-HT from the cell body of the serotonergic cerebral giant cell. Individual events are shown on an expanded timescale. In (B) examples of simple and multiple events with associated first derivatives (below). Line above differential is 4.5 × rms of the noise. In (C) a schematic of a vesicular event defining time constant of decay (τ) and the charge (Q). Q was calculated from the area of the vesicular event and τ was obtained by fitting an exponential function to the decay phase of the event.

vesicular release events is due to both molecular diffusion and re-uptake.[23]

Rigorous interpretation of the form of the vesicular release traces in intact neuroneal systems is more complex than for chromaffin or PC-12 cells since local concentration profiles are a function of both the release process and re-uptake, both of which are modulated by sensory processes and inhibitors. These processes are discussed in detail by Wightman *et al.* in the context of stimulated dopamine overflow in rat brain slices where re-uptake is modelled by Michaelis–Menten kinetics.[23] Re-uptake can therefore be approximately characterised by essentially first order kinetics (provided transporters are some way from being saturated, as expected) and this would lead to an exponential decay in the concentration with time. The form of decay due to diffusion depends on the geometry. If the electrode–membrane distance is large compared with the diameter of the electrode, the vesicle will behave as a point source and concentration will fall as $t^{-\frac{1}{2}}$. However, if the electrode–membrane distance is small compared with the diameter of the electrode (as is the case for these experiments), the vesicular release can be approximated by a line source, *i.e.* a Dirac function. In the absence of the electrode there is a well known result obtained by integration of point sources along the line giving the concentration at the origin falling as $1/t$.[24] This time dependence is also preserved for non-infinitesimal cylindrical release normal to the plane.[25] Using first order kinetics to model the re-uptake and a Dirac function to model the vesicular release and diffusion limited oxidation at the electrode surface (a short distance from the cell membrane) leads to predicted exponential dependence of the current with time for both fast re-uptake and blocked re-uptake at long times.[26]

Clearly, all traces will contain both elements though limiting cases dominated by either molecular diffusion or re-uptake kinetics are possible. The vesicular events described here were best fitted to an offset exponential, as shown for a representative trace in Fig. 2 where the comparison with $-\ln(t)$ is also shown. The fitting function is:

$$i = i_0 + A\exp\left[\frac{-t - t_0}{\tau}\right]$$

where t_0 is a constant, not a fit function. Simple visual inspection shows that the exponential fit is substantially better. Statistical comparison with other functions confirms this observation (χ^2 = 563 for the exponential fit, *versus* 14 875 for $-\ln(t)$, 14 874 for $1/t^{\frac{1}{2}}$ and 14 872 for $1/t$). It is therefore reasonable to extract the time constant of decay from individual events and interpret this as characterising the kinetics of re-uptake.

Clearly, the larger the electrode, the greater the likelihood of more than one vesicle fusing with the microelectrode surface during a "single event". However, in our hands, smaller electrodes show poorer signal to noise, consistent with noise pick-up from external sources (pumps, lights *etc.*) being the dominant noise source. Since electrode fouling is an ec process, smaller electrodes foul more rapidly at any given concentration. In order to identify the optimum electrode size for detecting 5-HT vesicular release, we compared representative traces obtained from 7 and 30 μm carbon fibre microelectrodes

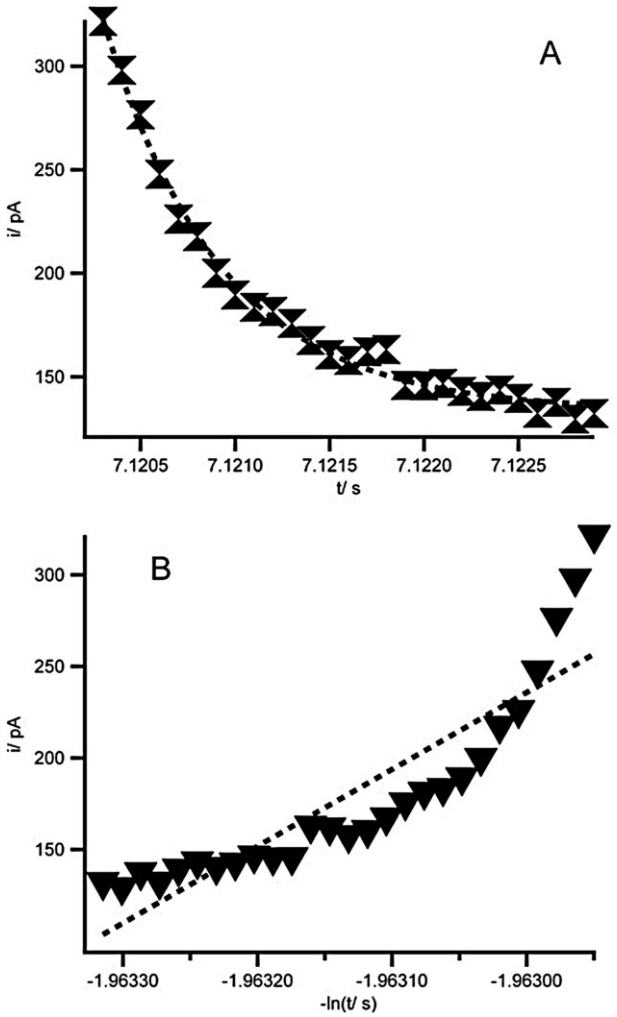

Fig. 2 Comparison of fits for a typical vesicle trace. The dashed line in (A) shows the fit to the exponential function: $i = i_0 + A\exp\left[\frac{-t+t_0}{\tau}\right]$, markers show the experimental data. The dashed line in (B) shows a linear fit of i to $-\ln(t)$, markers show the experimental data.

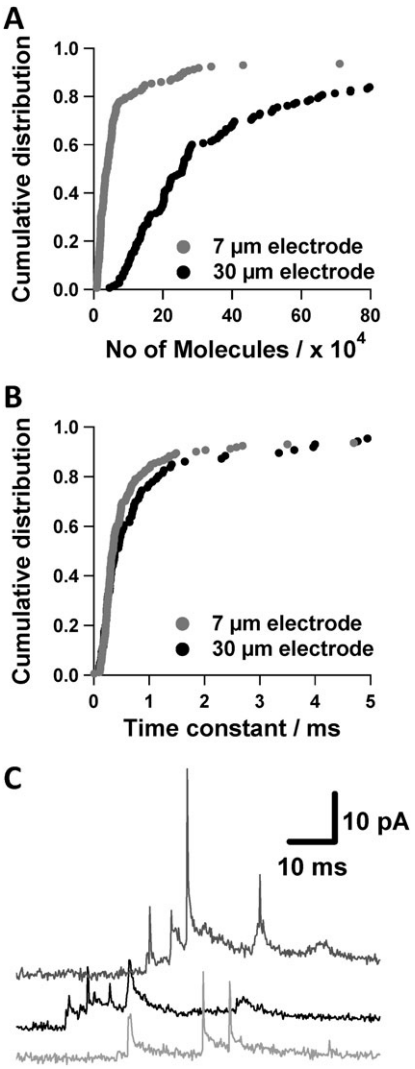

Fig. 3 Comparison of 7 and 30 µm carbon fibre electrodes for the detection of 5-HT release. Cumulative distribution curves of (A) Q (expressed as the number of 5-HT molecules) and (B) time constants of decay (τ) are shown. In (C) sample events obtained from the 30 µm electrode are shown.

examples of which are shown in Fig. 2. In Fig. 3A, cumulative distribution curves of Q (plotted as the number of 5-HT molecules) are shown for the two electrode sizes. There was a 5 fold increase in the median number of 5-HT molecules released per event on the 30 µm electrode ($p < 0.01$, one way ANOVA). However there was no significant difference in the time constant of decay, τ (Fig. 3B, $p = 0.75$). Median values for 7 and 30 µm electrodes were 0.35 and 0.41 ms, respectively. This would suggest that the amplitude of events on the 30 µm electrode is greater than that on the 7 µm. Sulzer and Pothos have shown that the likelihood of two events occurring at the same time is highly unlikely.[27] However on the 30 µm electrodes additional events are seen to occur during the decay phase of the initial event, increasing the number of multiple events observed; see Fig. 3C where events are shown to be occurring close together on the 30 µm electrode. Due to the high fraction of 'complex' events observed on the 30 µm electrode, and the higher probability of 'simple' events observed on the 7 µm electrode, the 7 µm carbon fibre disc electrode was chosen to study changes in vesicular release during ageing.

Effects of age on spontaneous vesicular 5-HT release

Attempts to evoke release from the CGC using either a high Ca^{2+}/high K^+ solution or by depolarising the cell using current injection *via* an intracellular microelectrode proved unsuccessful (data not shown). The data presented therefore represent spontaneous release events. Although there were no clear age differences in the percentage of preparations in which spontaneous release could be recorded the cumulative distribution curve of Q (plotted as the number of 5-HT molecules) for both young ($n = 390$ events) and old ($n = 217$ events) animals showed that ageing was associated with an increase in the median number of 5-HT molecules released per event from 34 000 molecules in the young to 73 000 molecules in the old ($p < 0.001$; Fig. 4A). This increase in the number of molecules of 5-HT detected per event in older animals could be due to a variety of factors: (i) increased amount of 5-HT released per quantal event, (ii) loss in the

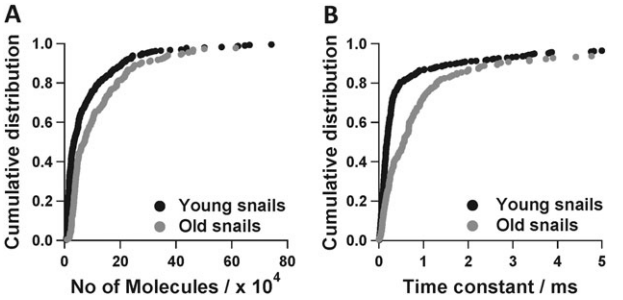

Fig. 4 Increasing age is associated with an increase in the size of the vesicular release event. Cumulative distribution curves of (A) Q (expressed as the number of 5-HT molecules) and (B) time constants of decay (τ) are shown.

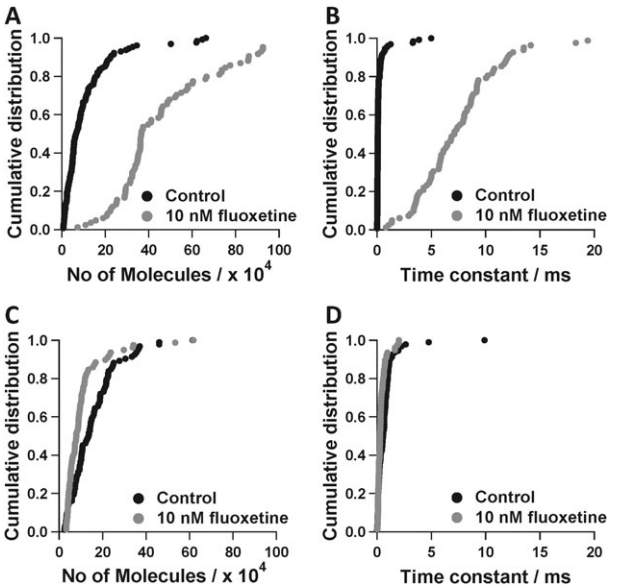

Fig. 5 Investigation of the effects of fluoxetine on vesicular release in young and old animals. In (A) and (C) cumulative distribution curves of Q (expressed as the number of 5-HT molecules) are shown and in (B) and (D) time constants of decay (τ) are shown. Responses from young neurones are shown in (A) and (B), whilst responses from older neurones are shown in (C) and (D).

function of the serotonin transporter (SERT), thus reducing clearance or (iii) loss in the activity of presynaptic autoreceptors, which are essential for regulation of the 5-HT released. Chromatographic studies have shown alterations in 5-HT levels and decreases in levels of the metabolite 5-hydroxy-indoleacetic acid (5-HIAA) with age.[14] Similarly, radio labelled 5-HT assays have shown decreases in clearance in various brain regions with increasing age [unpublished data].

From our experimental data we can study if there are any age-related alterations in the clearance of 5-HT by studying how τ changes. The cumulative distribution curve for τ is shown in Fig. 4B. Median τ increased significantly from (young) 0.26 ms to (old) 0.89 ms ($p < 0.001$). This significant increase in the median τ suggests reduced clearance in older animals, and thus a loss in the function or activity of SERT in older animals.

To investigate the involvement of the serotonin transporter, SERT, in the age-related increases in τ, we used the selective SERT blocker fluoxetine (Prozac) in young and old animals. In Fig. 5, the responses for Q and τ are shown for young and old animals in the presence and absence of 10 nM fluoxetine. Application of fluoxetine to the CGCs of young animals significantly increased Q (Fig. 5A) and τ ($p < 0.001$; Fig. 5B). This result is as expected as the clearance of 5-HT is blocked, 5-HT is left to diffuse into the extracellular space, resulting in increased values of τ. Similar responses have also been observed in a variety of other biological systems where fluoxetine was utilized.[28,29]

The increase in Q observed in the presence of fluoxetine (Fig. 5A) confirms that the vesicular events detected are due to serotonin, as the drug is a blocker of SERT which selectively clears 5-HT from the extracellular space. In the old animals, application of 10 nM fluoxetine caused a decrease in Q ($p < 0.01$; Fig. 5C) and also a decrease in τ ($p < 0.01$; Fig. 5D). The decrease in Q suggests that release events in fluoxetine-treated old neurones were not significantly different to those recorded from young untreated controls. Therefore, in *Lymnaea*, fluoxetine appears able to reverse an age-related change in the properties of the release events. While we have no clear explanation for this observation, the data suggest that the increase in the size of the release events seen in the untreated old animals was not simply due to a decrease in the number of 5-HT transporter proteins, but suggested either a change in the functional or structural properties of the protein with increasing age, or some additional effects of fluoxetine independent of its ability to block re-uptake.

Measuring NO release

In an attempt to evoke release of NO from the cell body of a single neurone, many approaches were studied as published previously.[20] A chemical stimulation using high Ca^{2+}/high K^+ HEPES-buffered saline increased NO production and release presumably by elevating cytosolic calcium concentrations. Fig. 5A shows that following the perfusion of the CGC cell body with high Ca^{2+}/high K^+ HEPES buffered saline, reproducible changes were recorded in the holding current of the electrode that were consistent with the neurone releasing NO. The response in Fig. 5A shown in grey shows a measurement conducted from a non-nitrergic (not releasing NO) neurone cell body, where no systematic changes in current were observed. Responses occasionally showed an initial decrease in the holding current, prior to the positive going waveform (see Fig. 6), even when electrodes were placed far away from the CNS tissue. This clearly is not part of the NO response and most likely represents transient changes in the double layer caused by the ionic superperfusate hitting electrode surface leading to transient capacitive currents as the ionic composition alters.[20]

The nitric oxide electrode was held at a potential which can also easily oxidise other neurochemicals present within the neurone, such as dopamine and serotonin. However we have previously shown[20] that the NO electrode shows excellent selectivity against other neurochemicals known to be present within the biological matrix and also against NO oxidative

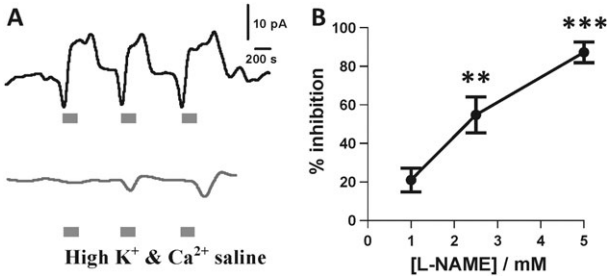

Fig. 6 Monitoring nitric oxide release. In (A) reproducible responses of NO release were observed from the CGC neurone following stimulation using a high Ca^{2+}/high K^+ HEPES buffered saline. When similar measurements were made from a non-nitrergic neurone (grey line), no responses were observed. Bar indicates the duration high Ca^{2+}/high K^+ HEPES buffered saline was superperfused over the cell body. In (B) a dose response curve showing the relationship between the concentration of L-NAME and the % inhibition of NO release from a young CGC neurone.

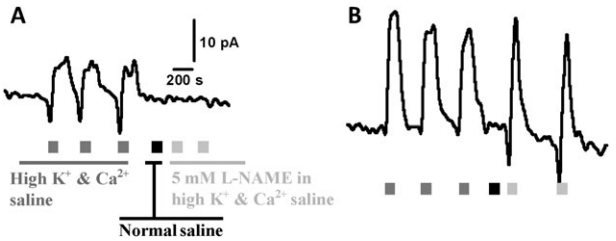

Fig. 7 NO responses observed from young and old CGC neurone cell bodies from the intact CNS. Measurements from young neurones are shown in (A) and responses from old neurones are shown in (B). The dark grey bar indicated the duration high Ca^{2+}/high K^+ HEPES buffered saline was perfused, whilst the black bar indicated the duration normal saline is superperfused over the cell body. The light grey bar indicated the period 5 mM L-NAME in high Ca^{2+}/high K^+ HEPES buffered saline was perfused over the neurones.

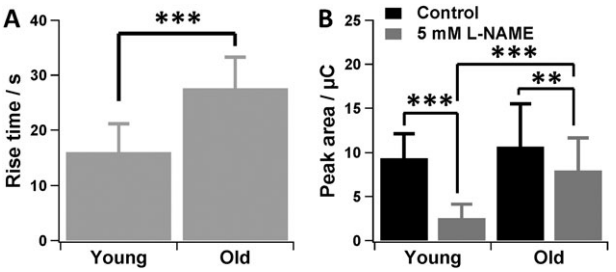

Fig. 8 Age-related changes in NO signalling. In (A) changes in rise time from NO release responses are shown, whilst in (B) the peak area responses are shown in the presence and absence of L-NAME. Where responses are shown as mean ± st. dev., $n = 22$ old CGC neurones and $n = 17$ for young CGC neurones. $^{**}p < 0.01$ and $^{***}p < 0.001$.

by-products such as nitrite. In addition, due to the time domain of sampling, we did not observe any vesicular events due to 5-HT release. These results suggest that NO is being detected at the electrode which is consistent with observations using NADPH-d staining or immunohistochemistry which suggested these neurones were capable of producing NO.[15,16]

The selectivity of the electrode for NO has already been described[20] however to further confirm that we were recording NO release the sensitivity of the evoked response to the selective NOS competitive inhibitor L-NAME was examined (Fig. 6B). Three concentrations of L-NAME were examined (1, 2.5 and 5 mM) and the percent inhibition of NO released was recorded. As the concentration of L-NAME was increased the amplitude of the evoked response was reduced. 1 mM L-NAME was capable of reducing the evoked response by 16.1 ± 6.2% ($p < 0.01$, $n = 3$, mean ± st. dev.) for the CGC cell. However 5 mM L-NAME inhibited evoked responses by 87.2 ± 5.4% for the CGC neurone ($p < 0.001$, Fig. 5B).

L-NAME has been widely used as an inhibitor to block the production of NO in HUVEC cells[30–32] and neuroneal tissue.[33] In molluscs, high concentrations of L-NAME were required to block NOS activity due to the presence of high intracellular concentrations (6 mM) of L-arginine in NOS-containing neurones.[34] Our results showed that a concentration of 5 mM was sufficient to see more than 80% block of NO release.

Age-related changes in NO release from CGC neurones

Representative experimental responses obtained from young and old CGC neuroneal cell bodies following superperfusion of various treatments are shown in Fig. 7. From the 36 old neurones measured of each cell type, only 22 CGC neurones were responsive. However no differences in the % of responding cells were observed between the two different age groups when measurements were carried out from the CGC neurone.

Increasing age was associated with significant increases in the rise times of the evoked responses. The rise time increased from 16.1 ± 2.6 s to 27.6 ± 5.7 s with increasing age ($p < 0.001$, Fig. 8A). The rise time (R_t) is an indication of the time taken for NO production to reach a maximum rate. R_t is affected by an interaction of NO release with secondary molecules or loss within other cells or tissue. This increase in rise time may be due to reduced efficiency of the NOS enzyme with age, but may also be due to increases in secondary molecules, such as superoxide, that reduce the levels of NO detected at the electrode by interacting with NO production or by chemical reaction to form peroxynitrite/peroxynitrous acid.

No differences were observed in the peak area of the response for CGC neurones from young and old animals (Fig. 8B). This would suggest that there was no difference in the amount of NO released in the two age groups studied. This is qualitatively different from the age-related changes in extracellular 5-HT (Fig. 4), where clear increases are observed which may have consequences on the behavioural output from the CNS. These data demonstrate that the composition of the signalling molecules released by a neurone can be significantly altered by subtle changes in the properties of key proteins that regulate the signalling process.

The effect of the NOS inhibitor L-NAME was investigated as a function of age. L-NAME (5 mM) decreased the peak area in younger neurones ($p < 0.001$) to a greater extent than in older neurones ($p < 0.01$). However there was a significant reduction in the ability of L-NAME to inhibit NO release in older neurones compared to younger neurones ($p < 0.001$, Fig. 8B) which may be due to either: (i) increases in expression of another isoform of NOS, (ii) loss of molecular specificity of

the active site and (iii) loss of function of intracellular enzymes. Korneev *et al.* have suggested that there is the possibility of more than one isoform present within the CGC;[35] however the concentration of L-NAME used here is known to be non-selective for all isoforms. Loss in the specificity of the active site may be a plausible reason, but this may also have a consequence on the conversion of L-arginine to NO and thus may have caused a reduction in the NO signal. The most plausible explanation for such alterations in the influence of L-NAME between young and old neurones is due to losses in intracellular enzymes. For L-NAME to be active, the ester group is cleaved by intracellular esterases in neurones which help trap L-NAME inside the cell. This would allow L-NAME to freely move inside and out of the cell in older animals, whilst being concentrated to cause inhibition in younger animals. Losses in the levels of esterases in older neurones have been observed previously.[36]

Alterations observed with ageing

Microelectrode amperometry provides a unique approach to studying the dynamics of neurotransmitter release in physiological preparations. By using two different microelectrodes to monitor key neurotransmitters present within a neurone, we can investigate how release profiles of signalling molecules can change with increasing age. 5-HT levels were elevated with increasing age, whilst NO levels were unaltered. These results suggest that age differentially affects these two signalling molecules that are released from the CGCs. As these neurones have previously been shown to be involved in regulating feeding behaviour[37] and learning and memory[38,39] the age-related deficits seen in these two behaviours may, at least in part, be due to these observed changes in signalling.

From both studies, losses in key proteins resulted in changes in signalling activity. Serotonin signalling is elevated due to losses in clearance by SERT. Decreases in esterases most likely explain why L-NAME activity in older neurones is reduced compared to younger neurones. These results are consistent with the hypothesis that ageing causes changes in the function of key proteins and that these phenomena lead to changes in a variety of cell functions such as signalling. These changes may also contribute to the age-related reduction in the connectivity between neurones in this system[14] and age-related changes in behaviour.[21,40,41]

Conclusions

We have described the application of two microelectrodes for the detection of serotonin and nitric oxide from an individual neurone in intact CNS. Age-related changes were observed in both transmission systems. Elevated levels of serotonin were observed due to reduced clearance by SERT. Although there were no changes in the total amount of NO released, the ability of L-NAME to inhibit NOS was reduced in older neurones. This was most likely due to losses in esterases within the neurone cell body. Overall microelectrode investigations have been able to identify age related changes in key protein function, which have led to alterations in signalling from the neurone.

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