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In Vivo Voltammetry: Promise and Perspective

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1. INTRODUCTION

In the brain, release of neurotransmitter candidates has been measured by a number of techniques, often perfusion-based. The push-pull cannula[71] is a method in which fluid is passed down one cannula and collected in another surrounding cannula. Any released neurotransmitter is collected in the perfusate for subsequent assay. The dimensions of the cannula determine the size of the structure that can be examined and mean that tissue damage is a potential problem[15,39,171]. When the ventricles are perfused[16], this is less of a problem. However, the ventricles bathe large areas of the brain and thus anatomical selectivity is lost. The cortical cup[56] is another variation of the same methodology in which the exposed cortex is superfused.

Recently, cerebral dialysis has been introduced[173]. This is, more or less, a push-pull cannula enclosed within a semipermeable membrane. Perfusion fluid does not come into direct contact with the brain. However, its physical dimensions are similar to those

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of the push-pull cannula and thus tissue damage remains a problem.

All perfusion techniques are indirect. Any compound collected in the perfusate must be analysed 'off-line'. Direct measurement of neurotransmitter release in vivo, without preparation of samples, was the objective to which voltammetry was directed.

2. VOLTAMMETRY

Polarography was discovered in 1924 by Heyrovsky[89] who found that current at a mercury electrode was not directly proportional to the applied voltage but appeared to be determined by the oxidisable chemicals in the solution. The extra current, above the predicted value, was proportional to the concentration of oxidisable species in the solution. By application of a suitable voltage, the current could be used to measure the concentration of oxidisable or reducable compound at the electrode surface. This science is called polarography when performed at the mercury electrode. Voltammetry is the same technique performed at all other types of electrode[2]. Normally the voltage is applied via an auxiliary electrode. A reference electrode, normally silver/silver chloride (Ag/AgCl) or saturated calomel, monitors this voltage. The voltage applied to the working electrode is maintained using feedback from the reference electrode to the auxiliary electrode. This provides potential control (the potentiostat).

When a voltage is applied to the potentiostat, the current flow through the working electrode consists mainly of two components: charging current and faradaic current. Charging current is due to charging of the electrical double layer of the electrode surface and is unrelated to redox processes. Faradaic current is due to oxidation and reduction processes. Most voltammetric techniques are directed towards maximising faradaic current and minimising charging current. This can be achieved by various approaches: very small electrodes, a few micrometers in diameter, have good mass transport characteristics and thus high sensitivity[72]. Slow voltage scan rates give higher faradaic to charging current ratios (faradaic current is proportional to the square root of the scan rate whereas charging current is directly proportional to scan rate[22]).

The voltage input waveform also determines the faradaic to charging current ratio. The simplest waveform is a voltage pulse. The voltage is increased instantaneously to a potential sufficient to oxidise the compound under investigation and held normally for one second. The current shows a sharp transient followed by decay to a steady level. The current is measured immediately before the end of the pulse. The advantage of this is that, following application of a voltage pulse, the charging current declines more rapidly than the faradaic current[22]. The later the sampling period the less charging current corrupts the faradaic current. This technique is called chronoamperometry. It gives accurate measurements of the concentration of electroactive species, but no identification.

Another approach is a linearly increasing voltage ramp. Faradaic current does not occur until the voltage reaches the oxidation potential of the compounds in solution. At these voltages the current rises to a plateau (or a peak at high voltage scan rates). If the species oxidise at sufficiently different potentials two distinct plateaux or peaks occur. This technique (linear sweep voltammetry) can provide information of the concentration of the electroactive species and some identification of the compounds. An extension of the technique is to follow an oxidation sweep with a reduction sweep. Compounds that are oxidised on the initial scan can be reduced on the reverse scan. The magnitude and potential of the reduction current gives further information about the compounds oxidised[1]. This technique is called cyclic voltammetry. In both linear sweep voltammetry and cyclic voltammetry the charging current is mainly proportional to the voltage sweep rate. It affects the lower limit of sensitivity more than with chronoamperometry.

Differential pulse voltammetry[137,162] combines aspects of chronoamperometry and linear sweep voltammetry. Small voltage pulses are superimposed upon a linear voltage ramp. The current is sampled immediately before a pulse and subtracted from the current at the end of the pulse. This pulse is applied about 3 times per second. The technique combines the main advantage of chronoamperometry (suppression of charging current) with the resolution of sweep voltammetry. Because it generates sharp peaks rather than the broad peaks or plateaux of linear sweep voltammetry, its resolution is very high[68].

The output current is virtually a differential of the linear sweep current, hence the name. An alternate approach is to differentiate the current output of a linear voltage sweep by using a resistor-capacitor network. This is called semidifferentiation and offers much the same advantages as differential pulse voltammetry[81,108].

Normal pulse voltammetry is a technique in which the voltage is a series of pulses of increasing size, returning to zero or a preset potential between successive pulses[62]. Current is measured as for chronoamperometry. The current output is like a series of points on a linear sweep voltammogram. The method has a good faradaic to charging current ratio, improved further by backstep correction.

All of the above methods have been used successfully in vivo. In addition, there are also anodic stripping voltammetry[142], steady state voltammetry[19], differential normal pulse voltammetry[80], differential double pulse voltammetry[106] and others, some suitable for in vivo usage, others purely analytical tools.

3. IN VIVO ELECTROCHEMISTRY

In the late 1960s, Ralph Adams began to divert the attention of his research group from aromatic electrochemistry to neurochemistry. He pointed out that the catecholamines were oxidisable and that their redox electrochemistry could be studied in vitro[156]. He hoped, since these compounds were neurotransmitters, that their release might be monitored in vivo by implantation of electrodes into brain tissue. Brain extracellular fluid is, in many respects, a good support electrolyte. Its resistance is low, its ionic concentration sufficiently high to prevent migration current and its pH is well buffered.

3.1. *Electrodes*

For in vivo use the working electrode (and to a lesser extent, the auxiliary and reference electrodes) must be miniaturised[3]. Most working electrodes used in electroanalytical laboratories are several millimetres in diameter, clearly unsuitable for in vivo use. Mercury electrodes too, as used in classical polarography, are clearly unusable in vivo. Most in vivo electrodes are carbon-based (carbon paste, epoxy, fibre or glassy carbon) although modified platinum has also been used in some experiments[106].

Carbon paste electrodes[111] have been popular because of their simplicity. They are made from teflon-coated silver wire. The teflon sheath is pushed down over the end of the wire to form a well which is filled with a paste made of graphite powder and either silicone oil or Nujol. The resultant electrode has a diameter of about 200 μm, low background current, and a flexibility which makes it less vulnerable to damage than more rigid types.

A variant of the carbon paste electrode is the carbon epoxy electrode[163]. In this the oil or Nujol is replaced by epoxy resin. Instead of the teflon-sheathed silver wire, a pulled glass micropipette is packed with the carbon epoxy mixture and then heat-cured. The tip of the microelectrode is cut to give an active surface of 50–100 μm diameter. These electrodes have found favour for chronic implantation where dissolution into surrounding brain tissue makes carbon paste electrodes perhaps less suitable. They are, however, more fragile than carbon paste electrodes.

Carbon fibre electrodes[6,12,69] are constructed from single (or multiple) 8 μm diameter carbon fibres enclosed in glass micropipettes. The protruding fibre can be cut to lengths of 500 μm downwards. Some cut the electrodes flat so that the active surface is a single 8 μm diameter disc[165]. These electrodes are very small (similar order of magnitude to neural elements) and induce minimal tissue damage. Their principal disadvantage is their frailty. They also deteriorate in vivo and are probably unsuitable for chronic implantation.

Glassy carbon electrodes[160,174] have been used only rarely[26]. Their resolving power appears limited in vivo[125].

Iodinated platinum electrodes have been tried briefly in vivo. However, their main advantage of enhanced electron transfer rates for ascorbic acid (AA) and the catechols is lost almost immediately upon exposure to brain tissue[106]. Their use has thus been severely limited.

3.2. *Measurement of exogenous compounds*

As soon as electrodes were implanted into brain tissue it became apparent that measuring dopamine (DA) release was over-optimistic with the existing methodology. Clark had implanted glassy carbon

electrodes into the anaesthetised cat brain in 1965. He examined the effect of oxygen, carbon dioxide and AA on the in vivo voltammogram[42,43]. He did not, however, observe any catechols or metabolites.

Most electrochemical experiments have been performed in rats. Within the rat, the corpus striatum has received by far the most attention. It is easy to find, rich in DA[136,161] and large enough to allow implantation of electrodes.

In the earliest experiments the endogenous voltammogram showed few features of interest. Most of the oxidation current appeared to be from AA[100]. These experiments concentrated on the interaction of exogenous compounds with brain tissue. It was shown that AA and DA could be detected in vivo following microinjection[127]. 6-Hydroxydopamine (6-OHDA), injected into the striatum was also measured by cyclic voltammetry[126]. 6-OHDA equilibrated rapidly with its oxidised quinoid form. This equilibration occurred whether the oxidised or reduced form was injected. The brain acted as a redox buffer maintaining 40% of the 6-OHDA in the quinoid form. The oxidation potential for 6-OHDA in vivo was similar to that observed in vitro.

Investigation of how the brain handles exogenous compounds is one area in which voltammetry may prove fruitful. Morgan and Freed (1981) showed that paracetamol entry into the brain could be monitored by linear sweep voltammetry[124]. Freed and Yamamoto (1981) have reported that paracetamol metabolites can also be measured[70]. Broxterman and Mos (1980) reported the effects of HA 966 on striatal DA metabolism. HA 966 is electroactive and, along with the effects on endogenous oxidation peaks, they saw the appearance of a peak for HA 966 oxidation[26]. Marsden[114,115], in his study of 5-HT in vivo, reported that the appearance of α-methyltryptamine in the brain could be monitored at +0.8 V vs Ag/AgCl. The pharmacokinetic applications of voltammetry[118] have, however, been relatively under-explored compared with neurotransmitter metabolism.

3.3. *Ionophoresis*

DA, NA and 5-HT are electroactive. One area of interest was the quantification of ionophoresis of these amines in vitro and in vivo. To calculate the dose of amine applied by ionophoresis, it was necessary to know the size of the drug barrels, the transport number of the amine, etc.[102]. Estimates were inaccurate and required the use of radioisotopes or other indirect methods. Electrochemistry offered the possibility of more direct quantification.

The majority of voltammetric techniques so far discussed use very slow scan rates (ca. 5–20 mV/s). Using carbon fibre microelectrodes, electroactive species could still be detected at very high scan rates (300 V/s) and the scan lasted only a few milliseconds. This allowed more frequent measurements. At these high scan rates, a large but constant charging current was observed. Small faradaic current increments could be detected by background current subtraction as for other voltammetric techniques[164].

Much of the resolution between compounds, seen at low scan rates, was lost. For ionophoresis of known compounds this was not a drawback. The technique could detect DA in vitro (as well as other DA agonists possessing a catechol nucleus) with good sensitivity[9]. Ionophoresis of DA in vitro also produced detectable concentrations at the electrode tip[10].

Ionophoresis of NA showed that a given ionophoretic eject current usually gave rise to a higher NA oxidation peak in vivo than in vitro, indicating restrictive diffusion. Sometimes the level was lower, implying active uptake of NA[13]. DA could also be monitored in vivo following ionophoresis. Used alternately as a neurophysiological probe and a working electrode it was shown that DA (10 μM tip concentration) depressed cortical unit activity[121]. By monitoring unit activity when performing voltage scans it was possible to check for changes due to electrolysis. The electrochemical waveform caused no direct stimulation of neurones[8] although it has been shown that the pulsatile electrochemical techniques can disrupt neuronal firing patterns[87]. Armstrong-James et al.[8] also reported that, in the absence of ionophoresis, they were unable to detect extracellular DA or other monoamines.

In a further study of 5-HT ionophoresis, it was seen that application of eject current did not result in instantaneous release of the drug[103]. At low eject currents (12.5 nA), the extracellular concentration of 5-HT took longer to reach steady-state levels than at higher currents (50 nA). The 'lag-time' also depends on the duration of the retain current previously applied. If 20 nA retain current was applied for one minute,

subsequent 25 nA eject current gave 70% of the steady-state 5-HT release after 15 s. Eight minutes of retain current meant that a subsequent 15 s of eject current gave no detectable 5-HT release at all. A similar phenomenon has been recognised for ionophoresis of substance P[84]. In the cortex a similar observation was made for NA[11]. To achieve a given extracellular level of NA, it was necessary to use higher ionophoretic eject current at the beginning of an application than at later times. This work shows the care necessary in interpreting results from short periods of ionophoresis.

3.4. *Stimulated transmitter release*

The first attempts at voltammetric measurement of transmitter release were performed with carbon paste voltammetric electrodes implanted into the lateral ventricles of anaesthetised rats and rabbits with stimulation of the nigrostriatal pathway[168,169]. The lateral ventricle was selected for these experiments because the working electrode could be removed and resurfaced between measurements and cerebrospinal fluid (CSF) could be withdrawn for chromatographic confirmation of voltammetric results. Stimulation evoked no immediate release of DA into the CSF. However, 20 min after a stimulation, the voltammetric signal rose sharply[96]. By cyclic voltammetry they were able to show that the species measured in CSF was electrochemically similar to homovanillic acid (HVA). This observation was verified by liquid chromatography (LC)[167]. In addition to identification of the released compound, LC also showed that in vivo voltammetry measured the correct concentrations (in this case, millimolar).

In the striatum, stimulation of the nigrostriatal pathway evoked immediate release of an electroactive species[61,104] which has been shown to be DA by pharmacological and electrochemical criteria[61,122]. The release was of short duration, being detectable in the extracellular fluid for only about 15 s. Its removal from the extracellular fluid was shown to be due to a low affinity, high capacity uptake system[63] that could be blocked by nomifensine[155] and methylphenidate[152]. These electrochemical events, occurring over a time scale of seconds, promise new information about the dynamics of DA release and uptake.

3.5. *Experiments with amphetamine*

The first attempts at measuring DA release directly were made with electrodes implanted in the striatum. Amphetamine, widely known to release DA was used as a test of the methodology. Initial results seemed encouraging. Using chronoamperometry, striatal oxidation current rose following amphetamine, consistent with DA release[46,78,90]. Although widely accepted that baseline chronoamperometric current was probably due to AA, it was considered improbable that AA could cause the increase in voltammetric current. Chey and Adams did, however, address this issue. They incubated rat caudate slices in vitro with amphetamine (5 μM–5 mM) and reported "no observable amphetamine-stimulated release" of AA (Chey and Adams, unpublished). Further support for this was obtained in vivo. Using iodinated platinum, Lane et al. had created an electrode that could resolve DA and AA oxidation peaks[109]. In the striatum they recorded 2 separate oxidation peaks at the appropriate potentials for DA and AA. When amphetamine was microinjected beside the electrode, there was a sharp increase in the 'DA' peak without any change in the 'AA' peak[109]. These data implied that AA did not account for the increased current following amphetamine.

Gonon and coworkers showed that, by electrochemical treatment[79], pyrolytic carbon fibre electrodes could resolve AA from DOPAC in vivo for 3 h or more[76]. After amphetamine, the DOPAC peak height decreased and the AA peak increased in both anaesthetised and conscious animals. They concluded that the increase in striatal AA following amphetamine explained the increases in oxidation current observed with the non-selective voltammetric methods.

Dayton et al.[55], using pulse voltammetry at carbon fibre disc electrodes, also showed that amphetamine increased the striatal AA oxidation signal. O'Neill et al.[134] similarly found an amphetamine-induced increase in their striatal AA peak.

The voltammetric data have now been supported by other techniques e.g. cerebral dialysis[45] and push-pull cannulae[66,148], which confirm that extracellular striatal AA does indeed increase following systemic amphetamine administration. This has had various repercussions. Above all it has shown the importance

of identifying electrochemical signals. A battery of pharmacological tests is required. The amphetamine experiments also emphasised the value of electrodes that were capable of resolving AA from other electrochemical signals.

3.6. *Surface modified electrodes*

Although some groups have concentrated on AA, others have tried to remove it from voltammetric signals. One approach has been modification of the working electrodes to suppress AA oxidation or shift it to a potential at which it does not interfere.

Although Lane et al.[109] had shown that this was feasible, the platinum electrodes were unstable and resolution of AA and catechol oxidation persisted for only a few scans in vivo[106]. The first workable solution to the problem was reported by Gonon's group[79] who showed that, with electrical pretreatment and the use of differential pulse voltammetry, their carbon fibre electrodes could separate AA and catechol oxidation. In the striatum, they reported two oxidation peaks at −50 mV and +100 mV vs Ag/AgCl, respectively. The first peak occurred at the same potential as AA in vitro; the second was at the catechol oxidation potential. They ascribed these peaks to AA and DOPAC, respectively, on the basis of pharmacological manipulations.

A similar separation of AA and catechol oxidation peaks has been reported for carbon fibre electrodes by Plotsky[138]. He achieved this by anodic etching of the electrode in chromic acid. Falat and Cheng[64] reported that electrical pretreatment of carbon epoxy electrodes gave similar good separation of AA and catechol oxidation. As a result of adsorption, the electrodes were very sensitive to DA but unable to follow rapid changes in DA concentration.

An alternative solution to the interference of AA has been to prevent its oxidation rather than move it to a convenient potential. Nagy et al.[129] described a carbon paste electrode whose surface was surrounded by a dialysis membrane. Between the membrane and the electrode surface was a drop of ascorbate oxidase (AAOX) solution which oxidised the AA before it reached the electrode surface but had no effect on other electroactive compounds. The group also showed that AAOX could be attached to carbon-epoxy electrode surfaces with glutaraldehyde. The electrodes did not detect AA (140 μM) in vitro. In rat caudate slices potassium stimulation increased the electrochemical current at these electrodes, presumably by DA release. Milby et al.[119] had previously shown, in vitro and in vivo, that potassium ion stimulation could cause AA release from rat brain preparations and was therefore a potential source of interference at untreated electrodes in vivo. The principal drawback of the AAOX-based electrodes was their stability. The dialysis membrane variety lasted 4 days and the glutaraldehyde-linked type was useable for 10 days. The electrodes were slow to equilibrate (3 min to steady-state response) which limits their use for measuring very rapid transmitter release.

Another approach was the covering of graphite-epoxy electrodes with Nafion[73], a perfluorosulphonated polymer which repels anions but allows cations through. In vitro and in vivo, the electrode did not respond to AA or DOPAC, but measured DA well. As with the AAOX electrodes the response time was slow. Gerhardt et al.[73] pointed out that the time constant might be too slow for some potential scanning techniques.

Carbon paste electrodes offer much scope for modification. Blaha and Lane (1983) described an electrode in which the carbon powder was added to a 10% v/v mixture of stearic acid in liquid paraffin[20]. Stearate is an anion. The introduction of its carboxyl groups onto the electrode surface retards electron transfer from anions (DOPAC and AA). In vitro, the electrode showed good selectivity for DA over AA and DOPAC. In the rat striatum it gave no chronoamperometric response to i.v. AA although an unmodified carbon paste electrode responded well. Pargyline increased chronoamperometric current at the stearate electrode and decreased current at the normal electrode[21]. The electrodes showed good selectivity too when used with linear sweep voltammetry[172].

Other groups have used sodium dodecyl sulphate (SDS) as the anionic modifier[98,130]. SDS gave the same effect as stearate, i.e. an anodic shift of the AA and DOPAC oxidation potentials. However, despite the high oxidation potential of AA at SDS-modified electrodes, the DA oxidation peak height varied with the AA concentration[130]. This is the 'electrocatalytic' effect, whereby the oxidation current for DA is am-

plified by the AA concentration. Although electrocatalysis is often used in vitro as an analytical aid[60,157,159], in vivo its occurrence is undesirable.

The occurrence of electrocatalysis depends on the relative voltages at which AA and DA oxidise. When AA oxidises at a lower potential than DA[79], electrocatalysis cannot happen, whereas shifting AA oxidation to higher potentials allows it to occur. The reaction is due to the irreversibility of AA oxidation. Dehydroascorbate (DHA), the oxidation product of AA, hydrates in aqueous solution and cannot be reduced. When DA is oxidised by the working electrode to the orthoquinone (DOQ), some DOQ oxidises AA to DHA, at the same time reducing itself back to DA[166]. This DA is then available for electrooxidation again. In the presence of AA, a DA molecule may be oxidised more than once during a voltage scan, thus increasing the DA oxidation current. The extent of this depends on the duration of the measurement and the size of the electrode. Shorter electrochemical measurements reduce the extent of electrocatalysis[101]. At very small electrodes, a smaller fraction of the regenerated DA finds its way back to the electrode surface. Short electrochemical measurements at small electrodes greatly reduce the problem.

Although Newell and Colhoun showed that both SDS and stearate-modified paste electrodes did exhibit catalysis between DA and AA[130], Lane's group showed no effect[20,172].

Adams and Marsden[4] pointed out that "surface modification techniques are more art than science at present". Although, at present, there is no 'DA electrode' or 'AA electrode', systematic alteration of the electrode is a fruitful approach to the problem of selectivity in voltammetry.

3.7. *Identification of electrochemical signals*

Since the experience with amphetamine, it is clear that more than one approach is required to identify an in vivo voltammetric signal. The modification of electrodes to make them less sensitive to some compounds is a useful first step.

Most slow-scanning voltammetric techniques detect two or more peaks in vivo, the number depending on the voltammetric technique, the electrode type and the region into which the electrode is implanted. Lane et al.[107] showed, using linear sweep voltammetry in the striatum, that at a 10 mV/s scan rate, 4 peaks were detectable between 0 and +800 mV vs Ag/AgCl. At 150 mV/s scan rate there were only two peaks.

The electrode also determines the voltammetric response. Differential pulse voltammetry in the striatum showed 3 peaks between −100 and +300 mV vs Ag/AgCl with carbon fibre electrodes[51] but only two with carbon paste electrodes[24]. Linear sweep voltammetry with paste electrodes too shows the same[134].

Various strategies have been applied to ascertain the contributors to each of the peaks. A good initial approach is the microinjection of suspected candidates beside the working electrode[24]. The fact that a microinjected compound increases the height of an endogenous oxidation peak does not prove that it is a contributor. However, the reverse does apply: failure to match the oxidation of an exogenous compound with the endogenous peak means that it cannot be a contributor.

Reliance solely on microinjections to identify a peak has also caused mistakes. Until recently, it was thought that 5-hydroxyindoles contributed to the oxidation peak at +250 mV vs Ag/AgCl in the striatum observed with carbon paste electrodes. More recent data have shown that uric acid is responsible for this peak in vivo with carbon paste electrodes[132]. Uric acid also contributes to the proposed 5-HIAA peak at carbon fibre electrodes with differential pulse voltammetry[50]. These results stress the dangers of peak identification by microinjections.

The best identification of voltammetric signals is by pharmacological manipulation and selective lesions. Gonon et al.[76] showed that the striatal peak 2 (the proposed catechol peak) was not observable in rats whose nigrostriatal pathway had been destroyed by 6-OHDA. The AA peak in the striatum was unaffected by the lesion. Conversely, Lane et al.[107] reported that their first striatal peak (+210 mV vs Ag/AgCl) was unaffected by nigrostriatal lesion, indicating that catechols were only a small component of the signal.

Cespuglio et al.[38] showed that the proposed striatal 5-hydroxy-indole peak decreased by 65% following destruction of the median forebrain bundle by electrocoagulation or infusion of 5,7-dihydroxytryptamine. The catechol peak was less affected by the

chemical lesion than by coagulation.

A similar strategy has identified AA oxidation peaks. Lesioning is not feasible since AA is not wholly assoeiated with any single neuronal pathway. (Recent evidence has shown that striatal AA levels are at least partly controlled by the cortico-striatal glutamate projection[135].) An effective way of testing the AA contribution is to make the animals scorbutic. This cannot, however, be performed in rats. With only rare exceptions[123], rats can synthesize AA and do not need dietary intake. Guinea-pigs, in common with man and some birds, cannot synthesize AA[40]. Buda et al.[27] showed that the voltammogram in guinea-pig striatum was similar to that in the rat. After 24 day deprivation of dietary AA, the AA peak was undetectable. The catechol peak was unaffected.

Another approach is to remove AA from the voltammetric signals by enzymatic oxidation. Ascorbate oxidase (AAOX) is an enzyme which selectively oxidises AA to DHA, thus removing its contribution from a voltammogram. Brazell and Marsden[25] showed that microinjection of AAOX beside the electrode abolished the proposed AA oxidation peak without effect on the other peaks.

Having found that a peak is due to catechols or 5-hydroxyindoles, it is necessary to identify these catechols or 5-hydroxyindoles. Gonon et al.[75] showed, with differential pulse voltammetry at carbon fibre electrodes, that α-methyl-*p*-tyrosine and NSD 1015 both reduced the catechol peak in the striatum. The effect of NSD 1015 indicated that L-DOPA was unlikely to be the catechol measured. Pargyline almost abolished the peak, showing that the main catechol contributor was DOPAC. The contribution of DA to the peak was negligible in control animals. After pargyline treatment, a small DA peak could be detected[80] which increased following amphetamine or haloperidol. Interestingly, the identity of the catechol peak is not always predictable. When investigating noradrenergic metabolism in the locus coeruleus it was found, surprisingly, that the catechol peak was due to DOPAC and not, as might be expected, dihydroxymandelic acid or dihydroxyphenylglycol[29,77].

Acidic metabolites are also responsible for the 5-hydroxyindole peak detectable by differential pulse voltammetry. This was reduced by 70% in animals pretreated with *p*-chlorophenylalanine. NSD 1015 and Ro 4-4602 also decreased the peak by 50% or more. Clorgyline caused a similar decrease indicating that 5-HIAA constituted a major part of this peak. Thirty percent of the peak was not attributable to 5-hydroxyindoles and may reflect blood contamination[35] or uric acid oxidation[50].

Techniques which scan to high potentials have detected other peaks. O'Neill et al.[134] observed a peak at +370 mV vs Ag/AgCl in striatum but not hippocampus[83] which was proposed as homovanillic acid (HVA). Lane et al.[107] showed that 3 methoxytyramine also oxidised at this potential in vivo. However, as with the earlier peaks, it appears likely that the peak is due to an acidic metabolite rather than an amine.

At still higher potentials (+600 to +750 mV vs Ag/AgCl) another peak is often seen. This is the potential where the amino acids tyrosine and tryptophan oxidise in vitro. Possibly these amino acids or peptides containing them[17] contribute to this peak in vivo. However, the peak remains unidentified.

Pharmacological identification of peaks has shown that principally metabolites, rather than the amines, contribute in vivo. Despite this lack of immediacy in the responses there are (at least) 3 areas where voltammetry can provide valuable information: AA, DA and 5-HT.

3.8. *Ascorbic acid*

Amongst the most interesting consequences of the amphetamine experiments has been the investigation of AA in vivo by voltammetry[27,66,150,154]. AA shows regional variation in tissue concentrations[120,141] which is also reflected at the extracellular level[153]. It has been shown to interfere with DA 'receptor' binding in vitro[85,88,110] and to affect cerebral levels of amine neurotransmitters[92]. In turn, cerebral AA levels are affected by various neurotransmitters[158].

Wilson et al.[170] have shown that the release of AA by amphetamine depends on the site of application. Systemic or unilateral intranigral amphetamine or DA increased AA release in both striata. This contrasts with the effects of nigral amphetamine on DA release which showed reciprocal changes in ipsilateral and contralateral caudate nuclei[41]. Wilson et al.[170] also showed that infusion of amphetamine into the striatum did not cause striatal AA release, in agreement with Lane et al.[109].

It seems unlikely that AA is released concomitantly with DA from nigrostriatal nerve terminals in the caudate. Clemens and Phebus[44,45] showed, using dialysis and voltammetry, that systemic pergolide (a DA receptor agonist) increased extracellular AA in the striatum and spiperone (a DA receptor antagonist) blocked the effect. This indicates that there may be a dopaminergic component to the release of AA in the striatum but that AA release does not occur with DA simultaneously.

Much evidence has emerged from Fillenz's laboratory that striatal AA may be more closely involved with the excitatory amino acids glutamate and aspartate[133]. Striatal AA has been shown to exhibit a circadian rhythm[131] with peak levels at night. Lesioning of the corticostriatal pathway (a major glutamatergic projection) decreased the striatal AA oxidation peak and abolished the noctural rise[135]. Glutamate also released AA from striatal synaptosomes[67]. The absence of inhibition by glutamate antagonists implied that the effect was not receptor-mediated. Grunewald and Fillenz[82] showed that the response was blocked by inhibitors of high affinity glutamate uptake and thus appeared to be due to a 'heteroexchange' mechanism.

The increase in extracellular AA in the striatum does not occur in the hippocampus, for instance, where the AA oxidation peak is decreased following amphetamine[83] or in the olfactory tubercle where there is no effect[112]. Despite current interest in AA it is still not clear how it is released and with what (if any) neuronal systems it is involved.

3.9. *Dopamine*

Two principal areas have been investigated: correlation of behaviour with electrochemical responses and the investigation of drug-induced release, i.e. neurophysiology and neuropharmacology.

Stress (by restraint, tail pinch, electric shock to the tail or swimming in ice water) has been shown to increase DA-related electrochemical signals in the striatum[52,53,97]. The voltammetric changes were sharp and declined rapidly when the stressor was removed. Although AA oxidation could not be excluded from the basal voltammetric signals, the increases probably reflect DA-related changes. Tail pinch-induced gnawing, for instance, appears to result from DA release from nigrostriatal neurones[7]. Using a selective voltammetric method[75], it was shown that swimming stress increased the DOPAC oxidation peak[91] without any reported change in the AA peak.

Another area of physiological interest is the anterior pituitary where DA is believed to be a prolactin-release inhibitory factor (PIF). During suckling, prolactin is released, possibly due to removal of a tonic DA mediated inhibition. Plotsky, de Greef and Neill[139] implanted electrodes (carbon paste or fibre) into the median eminence and measured chronoamperometric and differential pulse voltammetric signals. Alpha-methyl-*p*-tyrosine halved the signal. Stimulation of the isolated median eminence by acetylcholine or electrical field stimulation dramatically increased electrochemical signals in the median eminence. Chromatographic analysis of the incubation fluid showed increases in DA, DOPAC and HVA but not AA.

Suckling (simulated by electrical stimulation of the mammary nerve for 15 min) caused a sharp fall in the electrochemical signal in the median eminence. Sham stimulation had no effect. Following stimulation there were unpredictable changes in the signals, usually an increase. The decline in electrochemical signal was supported by radioisotopic measurements which showed a decreased DA content in the pituitary stalk blood[140]. These results were consistent with the belief that DA acts as a PIF and that during lactation its release is decreased.

O'Neill et al.[131] reported that some peaks showed circadian variation. The peak at +370 mV vs Ag/AgCl in the striatum, purported to reflect HVA oxidation, was maximal at night. Parallel changes were seen in the AA peak. Baumann and Waldmeier (unpublished), reported that the DOPAC peak also increased at night. Curzon and Hutson[54] reported that the peak at the 5-hydroxyindole potential did not show a circadian variation in the striatum.

Although voltammetry has much to offer in correlation of behaviour with neurochemical changes, it is also suited to neuropharmacology. Using carbon fibre electrodes and differential pulse voltammetry, Gonon's group[28,76] showed that haloperidol (0.5 mg/kg) increased the DOPAC peak height in the striatum and nucleus accumbens. Louillot et al.[112], using the same technique, showed an increase in the DOPAC peak in the olfactory tubercle too following

haloperidol. These results were consistent with increased activity of DA neurones in response to DA receptor blockade. Albery et al.[5] and Lane et al.[107] showed that there was also an increase in the striatal HVA peak, further supporting the interpretation.

Interestingly, Buda et al.[28] reported that haloperidol did not significantly increase the DOPAC peak in the substantia nigra or ventral tegmental area. The simultaneous monitoring of drug effects in different areas thus allows new insights into their actions.

Maidment and Marsden[113] recently examined autoreceptor responses in the mesolimbic DA pathway. They microinjected DA or haloperidol into the ventral tegmental area. Haloperidol increased the DOPAC signal by 48% in the ipsilateral nucleus accumbens. DA decreased the DOPAC peak by 36% within 20 min, followed by a large increase above baseline levels.

Microinjection of drugs into specific regions of the brain allows their local effects to be studied. Sharp et al.[151] reported the effects of infusion of a thyrotrophin releasing hormone (TRH) analogue (CG 3509) into the nucleus accumbens or striatum on the DOPAC oxidation peak in those regions. They found that CG3509 increased the signal in the nucleus accumbens but not in the striatum. This supported the in vitro data from the same group[18].

Although selectivity remains a problem with catechol oxidation, voltammetry can provide insights into the release of DA in vivo. Selective electrodes are more necessary in this area than for AA or 5-HIAA.

3.10. *5-Hydroxytryptamine*

The 5-hydroxyindole peak has been the most studied voltammetric peak because of its clear resolution and size relative to the others. Recent evidence postulating a contribution of uric acid has, however, shown that caution is necessary in interpreting the results obtained with this peak.

Early experiments, as with DA, involved stimulation of 5-HT neurones. Wightman et al.[168] reported that electrical stimulation of the midline raphe nuclei released 5-HIAA into the lateral ventricles which was verified by L.C. Marsden et al.[117] reported that stimulation of the median raphe nucleus caused a sharp increase in hippocampal oxidation current without any effect on the striatal signal.

Using differential pulse voltammetry at carbon paste electrodes, Brazell and Marsden[23,24] reported an oxidation peak at the 5-hydroxyindole potential in striatum and frontal cortex. The peak was abolished by *p*-chlorophenylalanine[116] indicating that it was likely to be due to 5-hydroxyindoles.

Linear sweep voltammetry at carbon paste electrodes in the hippocampus has also shown a peak at the 5-hydroxyindole potential which was reduced by *p*-chlorophenylalanine and increased by *p*-chloroamphetamine[93,99] and by tryptophan[94]. The peak did not change however after pargyline. This was taken as evidence that 5-HT contributed to the signal as well as 5-HIAA. However, recent evidence[132] has shown that, at carbon paste electrodes, this peak is due to uric acid in the striatum. The same may also apply in the hippocampus. Carbon fibre electrodes are less sensitive to uric acid. Most (60–70%) of the striatal 5-hydroxyindole peak appears to be due to 5-HIAA[36,37] while uric acid accounts for 30%[50].

The technique of Cespuglio et al. (differential pulse voltammetry at electrochemically treated carbon fibre electrodes[35]) has been more successful than any other for measuring 5-HIAA and measurements have been made in striatum[30], cortex[32], spinal cord[143], CSF[86], suprachiasmatic nuclei[65], hypothalamus[14] and in raphe nuclei[34].

Cespuglio showed that the 5-HIAA peak fluctuated during the sleep–wake cycle of the rat. The largest peak occurred during waking, was smaller in sleeping rats and smallest during paradoxical sleep. The sleep patterns were monitored by electroencephalography. The changes in 5-HIAA peak height were observed in areas of 5-HT cell bodies and nerve terminals and were the same in each area studied: cortex and striatum[31], suprachiasmatic nucleus[65] and raphe nuclei (dorsalis and magnus[34,48], centralis, dorsalis, pontis and magnus[49]).

In all nuclei the fluctuations were small (ca 3% between successive measurements) but when an animal expressed one vigilance state for 15–20 min, the changes were more profound. The peak was higher during 'active waking' (when the rats were eating, grooming or washing), than in inactive consciousness[49]. Cespuglio et al.[34] pointed out the disparity between these results and the view that 5-HT is associated with sleep not waking states[74,95]. Cespuglio et al.[33], however, also showed that cells in the nucleus

raphe dorsalis showed higher firing rates when the animals were conscious and were almost silent during paradoxical sleep. These experiments with voltammetric and encephalographic recording represent an important area of research: the correlation of a behavioural pattern with a neurochemical parameter.

Differential pulse voltammetry has also been applied to anatomical investigation: the determination of the 5-HT innervation of the brain. Lamour et al.[105] showed a laminar distribution of the 5-HIAA peak in the rat somatosensory cortex. The largest peak was in the most superficial layers and decreased with depth. Electrical stimulation of the ascending serotonergic fibres at the lateral hypothalamus or in the dorsal raphe nucleus increased the peak height[144].

Crespi et al.[47] have mapped the raphe nuclei using the same method. The 5-HIAA peak was monitored during penetrations through raphe dorsalis, centralis, pontis and magnus. The peak height increased as the working electrode entered the nucleus. The peak was largest in nucleus raphe dorsalis, and consistent with biochemical data that 5-HT levels are highest in this nucleus[147].

In the dorsal raphe nucleus Echizen and Freed[57] have shown two oxidation peaks, using linear sweep voltammetry at carbon paste electrodes, one due substantially to 5-HIAA. These peaks responded to drug-induced alterations in blood pressure[58]. Hypotension, induced by nitroprusside, caused no change. Phenylephrine-induced hypertension doubled the 5-HIAA peak height which continued to increase following cessation of phenylephrine infusion. The same workers were also able to calculate the 5-HT turnover rate from the decline in the 5-HIAA peak following pargyline. In the dorsal raphe a value of 12.6 nmol/g/h was obtained[59].

5-HT is also present in the spinal cord and a 5-HIAA peak is observed in this area[145]. Electrical stimulation of nucleus raphe magnus increased the peak height[143,146]. This was not seen when 5-HT synthesis was blocked by *p*-chlorophenylalanine pretreatment.

In the striatum the 5-HIAA oxidation peak can be abolished by electrolytic lesion of the dorsal raphe nuclei[149]. However, transplantation of neonatal mesencephalic raphe nuclei into the lateral ventricles adjacent to the striatum restores the striatal 5-HIAA peak, indicating that metabolism of 5-HT occurred in the transplants[128]. Whether the metabolism is associated with release of 5-HT remains an open question. Nonetheless, voltammetry clearly provides a good tool with which to investigate this problem.

4. CONCLUSIONS

Throughout the course of this article I have attempted to draw attention to the pitfalls and successes of the in vivo voltammetry methods. Despite the drawback of imperfect selectivity, it is clear that voltammetry does have some advantages which make it the method of choice in certain areas of neurotransmitter research. The problem of selectivity is itself being addressed and the production of electrodes which can detect a single electroactive species (a DA electrode, for instance) is a promising area of research.

Probably the single most important feature of in vivo voltammetry is its ability to measure neurochemical events at the same time as the associated behavioural or neurophysiological correlates. Cerebral dialysis or push-pull cannulae, although capable of greater sensitivity and selectivity, require 'off-line' analysis of the neurochemicals by chromatography. The concentrations of metabolites or amines measured this way correspond to behaviour that the animal was expressing perhaps as much as an hour previously. Voltammetry gives the neurochemical measurement at the same time as the behaviour. The concentrations of amines and their metabolites measured by voltammetry and cerebral dialysis are in moderate agreement (voltammetrically determined levels are 2–10-fold higher than dialysis values) considering the major differences between the techniques.

Voltammetric electrodes are smaller than either push-pull cannulae or dialysis tubes. The largest voltammetric electrodes used today are no more than 200 μm diameter and the very small carbon fibre microelectrodes (8 μm diameter) are finding increasing favour with the in vivo electrochemists.

The advantages of very small measuring probes are clear. The smaller the probe the less tissue damage occurs, thus ensuring sampling from physiologically intact tissue. Simultaneous monitoring of cell activity at the tip of carbon fibre microelectrodes has shown that this is true. The other important gain from

the use of very small probes is the ability to sample from very small brain areas. This increases the scope of in vivo experimentation.

Voltammetry is also capable of measuring chemical events in the brain on a much shorter time scale than the perfusion-based methods. Push-pull cannulae and cerebral dialysis are limited by the duration of the sampling periods (5–20 min). Voltammetry has been used in vivo at measurement intervals as short as 250 ms. This is of value in the measurement of transient transmitter release (e.g. after electrical stimulation), where concentrations of neurotransmitter in the extracellular fluid can reach 50 μM for periods of about a second. In this application the much slower perfusion techniques are less helpful.

In vivo voltammetry, when used properly, is a powerful technique which can offer information about neurotransmitter release and metabolism unavailable to other methods. Its speed and ability to measure in small nuclei justify its position in the neurochemist's armoury.

SUMMARY

Dopamine, 5-hydroxytryptamine and noradrenaline are electroactive (oxidisable) neurotransmitters in the mammalian brain. Voltammetry, a technique which can measure the concentration of such compounds by their oxidation at an inert electrode, has been applied in vivo in the hope of measuring the release of these neurotransmitters without recourse to perfusion-based or post-mortem analyses. The measurement of neurotransmitter release is, however, complicated by the presence of high concentrations of other electroactive species (ascorbic and uric acids). Nevertheless, when used properly, with due emphasis on pharmacological identification of electrochemical signals, the technique can measure catechol and indole metabolites in vivo. Under certain circumstances the release of the catecholamines and 5-hydroxytryptamine themselves can be measured. The advantages and drawbacks of the voltammetric methodology are discussed.

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