Neuroprotection in an In Vitro Model of Ischaemia: Fast Cyclic Voltammetry Measurement of Dopamine Efflux During Oxygen and Glucose Deprivation
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Introduction
Cerebral stroke is a major cause of death and of neurological disability, but despite its impact on health, very few effective therapies are available. There is therefore a need for basic research exploring potential neuroprotective approaches to identify potential novel therapeutics. The majority of strokes are ischaemic and result from an interruption in arterial blood flow to the brain, causing restricted oxygen and glucose supply to tissue distal to the obstruction.

We have adapted the in vitro rat brain slice ischaemia model developed by Toner and Stamford [1] for use with mouse brain slices. Although a large amount of research on the mechanisms of ischaemic damage has focused on glutamatergic mechanisms, it is known that there is also massive release of monoamine neurotransmitters during ischaemia [see 1]. In particular dopamine levels achieved immediately following the onset of ischaemia have been shown to be neurotoxic and to contribute directly to the resulting cell death [2]. Thus, exposure of the brain slices to oxygen and glucose deprivation (OGD) evokes a substantial dopamine release, which can be measured by fast cyclic voltammetry (FCV). The neuroprotection offered by potential therapeutic agents is assessed by their ability to delay and/or reduce this dopamine efflux.

Epidemiological studies have established that pre-menopausal women, have a lower risk of stroke occurrence and experience improved recovery after stroke, but that risk increases after the menopause, coincident with a decline in circulating steroid hormones. Therefore it has been suggested that endogenous steroid hormones, including progesterone, may underlie this neuroprotection in females [3]. In terms of cerebral ischaemia, progesterone has been reported to reduce pathological damage due to ischaemia, supporting a neuroprotective role, possibly through an increase in GABAergic activity [4]. It is likely that progesterone increases GABAergic activity indirectly through metabolites that potentiate GABA-A receptor activity: in the brain, progesterone is rapidly reduced to the active metabolite allopregnanolone, which itself is more effective in reducing ischaemic brain damage [5].

We used FCV measurement in slices of mouse caudate nucleus in vitro during OGD-perfusion [6] to assess the neuroprotective role of allopregnanolone, and to investigate the role of GABAergic mechanisms in its action [7].

Methods
Male C57/Bl mice (10 – 16 weeks) were killed by cervical dislocation, and the brains rapidly removed and placed in ice cold artificial cerebrospinal fluid (aCSF).
Coronal 400 µm slices were cut on a vibrotome slicer, and transferred to aCSF, continuously aerated with 95% O₂/5% CO₂ at room temperature (21 ± 1 °C) [6,7]. After equilibration at room temperature (> 60 min), slices were placed in the recording apparatus, and perfused continuously with aCSF (33 ± 0.5 °C), flow rate 100 ml/h.

The FCV electrode comprised an 8 µm diameter carbon fibre which was inserted into a 10 cm length borosilicate glass capillary (OD., 2.0 mm; ID., 1.16 mm), and pulled to a fine tip in an electrode puller, such that the carbon fibre protruded from the pulled tip. The carbon was then cut to a length of 50 µm, under microscopic guidance. The electrode was inserted into the dorsolateral caudate nucleus and FVC recording started immediately, using a -1.0 to +1.4 V waveform (480 V/s, vs. Ag/AgCl) applied from a Millar Voltammetric Analyser. The signal was digitised and recorded using PClamp 9.0 software. An increase in the current signal at +600 mV, together with a corresponding reduction peak at -200 mV was characteristic of dopamine detection. At the conclusion of each experiment, the electrode was calibrated with a standard concentration of 10 µM dopamine, and this was used to convert current to dopamine concentration [6,7].

Slices were perfused with aCSF for 45 min, and then the perfusion medium was switched to OGD-aCSF for 15 min. In order to assess the effects of drugs, either allopregnanolone (0.1 or 1.0 µM) or allopregnanolone (1.0 µM) + bicuculline (100 µM) was added to both the normal aCSF and the OGD-aCSF perfusing the slices [7]. Dopamine release during OGD was assessed by (1) the time from switching to OGD-aCSF to the start of dopamine efflux (T_ON); (2) the time from the start of dopamine efflux to the peak of dopamine efflux (T_PEAK) and (3) the maximum concentration of dopamine released ([DA]_PEAK). The rate of change of dopamine during the OGD-evoked efflux (δ[DA]/time) was also calculated as [DA]_PEAK/T_PEAK.

Neuroprotection was deemed to have occurred if T_ON or T_PEAK increased, or if [DA]_PEAK or δ[DA]/time decreased [6,7].

aCSF composition was (mM): NaCl, 126.0; KCl, 2.0; KH₂PO₄, 1.4; MgSO₄, 2.0; NaHCO₃, 26.0; CaCl₂, 2.4; Glucose, 10.0; bubbled with 95% O₂/5% CO₂.

ODG-aCSF composition was (mM): NaCl, 126.0; KCl, 2.0; KH₂PO₄, 1.4; MgSO₄, 2.0; NaHCO₃, 26.0; CaCl₂, 2.4; Glucose, 2.0; bubbled with 95% N₂/5% CO₂.

Statistical analysis was by one- or two-way ANOVA, with post-hoc Tukey's multiple comparison test or Bonferroni test (respectively), where appropriate, using GraphPad Prism 5.

**Results and Discussion**

Perfusion with OGD-aCSF evoked a large efflux of dopamine from the striatal slices, starting 416 ± 53 sec after the onset of OGD perfusion (T_ON). The peak dopamine release (12.7 ± 2.3 µM: [DA]_PEAK) was achieved after a further 111 ± 45 sec (T_PEAK). Addition of allopregnanolone (0.1 or 1.0 µM) had no significant effect on either T_ON or T_PEAK (Fig 1a,b), but did cause a significant, dose-dependent decrease in [DA]_PEAK (Fig 1c). There was also a dose-dependent decrease in the rate of dopamine efflux, shown by δ[DA]/time (Fig 1d).
The GABA antagonist, bicuculline, had no significant effect on $T_{ON}$ or $T_{PEAK}$, either in the absence or the presence of allopregnanolone (Fig 1a,b). It did, however reverse the effects of allopregnanolone (1.0 µM) on $[DA]_{PEAK}$ (Fig 1c) and by $\delta[DA]/time$ (Fig 1d).

Therefore, the steroid sex hormone, allopregnanolone confers a degree of neuroprotection in this in vitro model of ischaemic stroke, which involves GABAergic mechanisms through GABA-A receptors [7].

![Figure 1. Changes in OGD-induced dopamine efflux.](image)

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References