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# Electrochemical Biosensors to Monitor Extracellular Glutamate and Acetylcholine Concentration in Brain Tissue

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## 1. Introduction

Glutamate (Glu) is considered the main excitatory neurotransmitter in the central nervous system and has a relevant role in several brain functions such as, synaptic plasticity, learning and memory processes (Bliss & Collingride, 1993; Fonnum, 1984; Nakanishi, 1992). Excessive release of Glu and over stimulation of its receptors has been associated with several neurological alterations including such as schizophrenia, Parkinson´s disease, stroke and epilepsy (Carlsson & Carlson, 1990; Klockgether & Turski, 1993; Dávalos et al., 2000, Morales-Villagrán, et al., 1996; Morales-Villagrán, et al., 2008). Another fast neurotransmitter; acetylcholine (Ach) has also been involved in learning and memory (Van Der Zee & Luiten, 1999), as well as a modulating agent in seizure activity, since positive stimulation of muscarinic receptor M1 could initiate generalized seizures in the basal diencephalon of the rat. (Cruickshank et al., 1994). Likewise, direct application of the agonist pilocarpine in brain tissue could produce seizures (Turski et al., 1989). To understand better the role of these fast acting neurotransmitters during normal or pathological activity, it is fundamental to precisely measure these compounds in brain tissue with high specificity and temporal resolution. Conventional analysis tools are all bulk-average-based methods, which means that the signals taken are an estimation of several physiological events that happen in a certain period of time, generally a few minutes. Microdialysis technique has been used to measure their extracellular brain concentrations; both in normal as well as in pathological conditions and these measurements are generally coupled to High performance liquid chromatography method (HPLC). However, such a technique is time consuming an especial training is required. Several methods coupled to enzymatic reactions that generate Hydrogen peroxide ($H_2O_2$) could be another alternative to measure some neurotransmitters, since numerous specific oxidases generate $H_2O_2$ as product, acting over certain neurotransmitters and avoiding the separation process. Measurement could be possible with fluorescence technique, like the use of the fluorogenic probe 10-acetyl-3,7-dihydroxiphenoxazine (AMPLEX RED), which reacts with $H_2O_2$ in the presence of horseradish peroxidase in 1:1 stoichiometry to produce a highly fluorescence product resorufin (Zhou, et a., 1997; Mohanty et al., 1997), which can be measured in a plate reader or in a fluorometer (In-vitrogen) but also a fraction collection of samples is required and the

time resolution will depend on the frequency of such a collection. Similarly, an alternative method to measure the $H_2O_2$ produced by enzymatic reactions is the use of an electrochemical detection system with about the same time resolution (Morales-Villagrán, et al., 2008a). To study the rapid neurochemical alterations during brain activity, the demand of rapid, reliable and sensitive determination of Glu and Ach is still a needing process. To overcome the necessity of fraction collection and to estimate these neurotransmitters concentration during the brain activity and near a real time resolution, the use of an enzymatic biosensor is merging.

These kind of biosensors are generally built with the same principle mentioned above, this is, they are constructed with specific enzymes that react with Glu and Ach to produce $H_2O_2$. In this chapter, it is described the process to built biosensors to monitor Glu and Ach during altered brain activity under the effect of 4AP.

## 2. Biosensor preparation

Electrochemical biosensors are prepared according to the method of Hu et al., (1994) with several modifications. A 125 m diameter platinum (Pt) wire 13 mm in length is inserted into a fused silica tubing (175 m i.d., 400 m o.d.) to strengthen the wire. To accomplish an electrical component a copper wire should be soldered to one end of the platinum wire covered with epoxic glue to insulate this terminal. Two mm of Pt wire extending outside the tubing is used as an active biosensor surface. Pt wire is covered with a film of a nafion solution at 5.0 %, the tip is heated for 3 minutes (~170 °C) and after ten minutes a film of cellulose acetate 5.0 % should be applied (acetone/ethanol 2:1). Biosensors are left at room temperature before fixing the enzymes. At this point, biosensors for Glu or Ach can be prepared using different enzymes, to determine Glu, twenty l of enzyme mixture are prepared in 20 mM of potassium buffer phosphate solution containing: 2% L-Glutamate oxidase, 0.3 % ascorbate oxidase, 5% porcine skin gelatin and albumin (2 %). This mixture was deposited on a glass surface and 5 l of 0.0625% glutaraldehyde was added and gently shaken, the platinum wire tips were immediately immersed deep into this solution. To determine Ach, a different mixture of enzymes is prepared containing 0.4% acetycholinesterase and 0.2% choline oxidase, albumin, ascorbate oxidase and porcine skin and glutaraldehyde are at the same concentration. Theses biosensors are dried at 37 °C for 30 minutes and are kept at 4 °C before using. Biosensors kept at this temperature condition retain their activity beyond than one moth.

Biosensor calibrations are carried out in a beaker, immersing a working electrode (biosensor), reference and counter electrodes in 20 mM potassium buffer solution with continuous shaking at room temperature. In this case, the formed cell was then connected to an electrochemical detector LC 4B (Bionalytical Systems), although it can be connected to any potentiostat. The electrical analogical signal coming out from the detector (voltage) can be digitized with a regular chromatographic software. It is necessary to reach a baseline value before doing any attempt of calibration. Different aliquots of Glu or Ach are added to an individual beaker to get a calibration reference. In fig 1 (A and B) calibrations for Glu and Ach are despicted. These biosensors were calibrated in vitro by increasing the substrate concentration in the range specified. Every step represents a single addition of Glu (A) or Ach (B). A linear regression analysis was done (inset) and the R-values calculated were 0.9

and 0.99, respectively. The total voltage scale corresponds to a generated current of 20 nA for Glu and 30 nA for Ach calibrations, corresponding to 50 nA/V. These results show that biosensors are adequate for their use *in vivo* conditions.

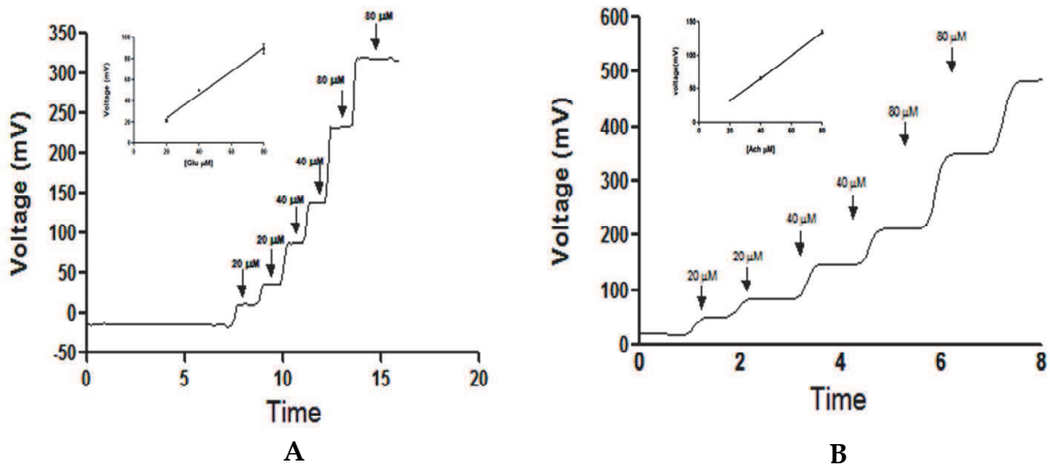


Fig. 1. Calibration curves for Glu (A) and Ach (B).

With respect to the speed of neurotransmitters measurement with these biosensors, time resolution was evaluated as the beginning of the response in each concentration until they reached a maximum value, this time was approximately of 20 seconds.

## 3. Animal studies

These biosensors can be used under anesthesia or in awake animals, as shown here. For Glu, biosensors were implanted into the cerebral cortex of rat pups (at three postnatal day) under anesthesia, in a three electrodes arrangement working, reference and counter, in order to accomplish an electrochemical cell *in situ*. Every biosensor must be calibrated before its use. Once the animal is recovered from anesthesia, the terminal of each electrode is connected to the potentiostat through a socket connector and after of an equilibration period to reach a baseline, the animal is ready to monitor the Glu extracellular concentration into the brain in any experimental condition. In the example showed here, the effect of subcutaneous monosodium glutamate administration in neonate rast (5mg/Kg of body weigh) was initially tested, resulting in a rise in extracelluar Glu concentration (Fig. 2A), this Glu elevation lasted approximately 20 minutes.

In previous work it has been demonstrated that in immature brain the blood brain barrier is not completely developed (Cernak, 2010) besides the high Glu concentration used is enough to disrupt the barrier due to an osmotic effect, similar effect has been found with the use of

manitol (Rapoport, 2000). Additionally in our previous work, it was showed that similar dose of monosodium glutamate can induce important rise in brain extracellular Glu concentration tested by internal biosensor and HPLC methods (Lopez-Perez et al., 2010). In order to induce seizures convulsion an additional systemic injection of 4-AP (3mg/kg of body way) was used, whose effect can be seen in the right side of the fig. 2A. It can be observed that after injecting the convulsant drug (50 min after starting recording) an increase in the extracellular Glu concentration is present that could be related to the intensity of seizure activity.

To test Ach biosensors, adult rats were used; they were also implanted with three electrodes, with the only difference that the working electrode was covered with necessary enzymes to determine Ach, and in this case the area of interest was the right thalamus. After a recovery period from anesthesia that lasted at least two hours, the animal is connected in a similar way as mentioned above to monitor extracellular Ach concentration during seizure activity, characterized by strong motor alterations like tonic-clonic convulsions. In the example showed here a baseline period of twenty minutes was recorded before testing the effect of 4-AP administration at 5 mg/kg of body (intraperitoneally). After the convulsant drug administration significant increments in Ach appeared that were also related with strong seizure behavior activity, this effect lasted about one hour (Fig. 2B) and finally the animal were euthanized with an intraperitoneal injection of pentobarbital. The examples showed here represent independent animal trials for Glu and Ach, respectively.

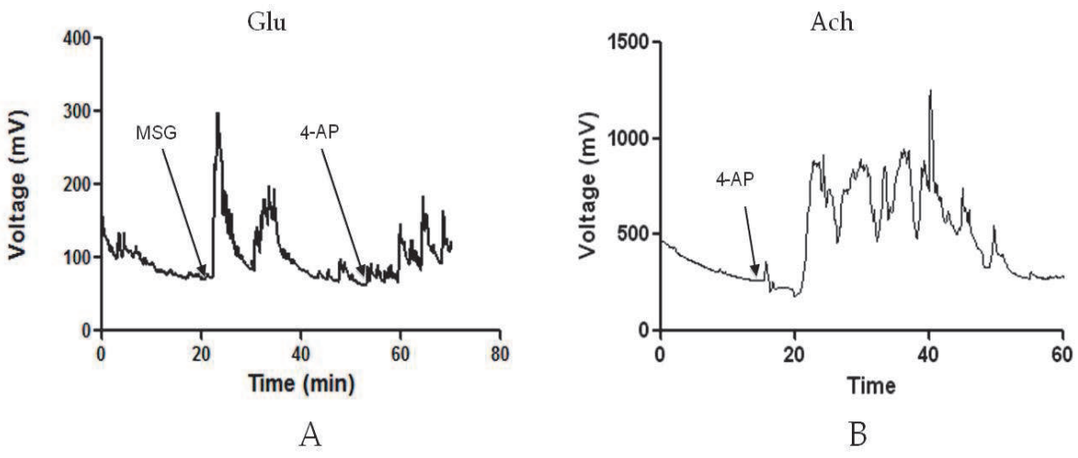


Fig. 2. Glu biosensor (A) and Ach biosensor (B) register during altered brain activity *in vivo*.

To evaluate the specificity of these biosensors, several controls can be run; one example is to test the response in vitro of these biosensors to other molecules that could produce a nonspecific signal, like monoamines and ascorbic acid, since without a good preparation a false positive result could appear. An example of such control for Ach biosensor is showed in Fig. 3A, the first two arrows represent additions of 300 µM concentration of ascorbic acid (Aa) and the two following of 80 µM Ach, they are represented by the next two arrows; it can be seen that this biosensor response specifically to Ach. Other way to test the specificity of a biosensor *in vivo* is to use one without enzymes in the cover; such naked or sentinel biosensor will not be able to sense any neurotransmitter concentration during any physiological conditions (Hascup et al., 2008) or calibration procedure. An example is showed in Fig. 3B, were a naked biosensor was inserted in the brain of an adult animal, this animal was treated with 4-AP, despite of the fact of appearance of strong seizure convulsion no any increase of Ach was detected with this biosensor. Spikes in graph B represent movement artifacts during convulsions. Similar analyses were done for Glu biosensors.

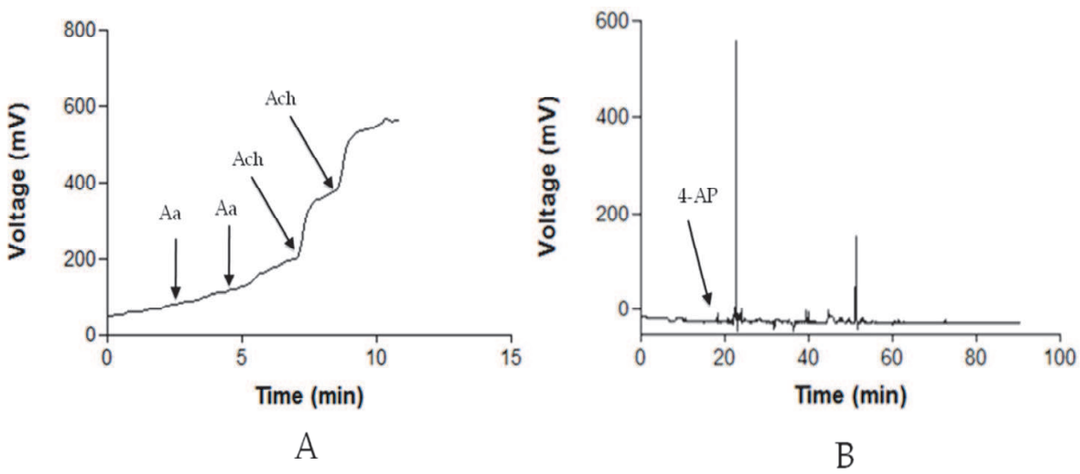


Fig. 3. Specificity test for Ach biosensor *in vitro* (A) and test of a naked or "sentinel" biosensor *in vivo* (B)

## 4. Conclusions

The use of electrochemical biosensors to monitor neurotransmitters concentration during normal or pathological activity in brain is an alternative approach that is gaining new users,

besides, different strategies to fix enzymes over several substrates are merging, like the use of sol gel derivates or other casting materials (Sakai-Kato & Ishikura, 2009; Hyun-Jung et al., 2010). This is a very important issue; this is trying to get biosensors that last active for more prolonged periods, which could overcome the necessity to monitor the neurotransmitter concentration for prolonged time or improving the way of fixing the necessary enzymes with more molecular movements that could allow such enzymes have more activity, since in general a fixed enzyme protein decreases its activity. Recent advances in the use of gold nanoparticles due to their increased surface area to enhance interactions with biological molecules, geometric and physical properties make them another alternative to prepare biosensors (Yang et al., 2009). With the procedure used here to monitor Glu and Ach it is shown that it is possible to evaluate the role of these fast neurotransmitters during seizure activity, since the increased release of these compounds have been related with the presence of a convulsive state, these neurotransmitter alterations have been determined with other methods, like microdialysis coupled to HPLC and pharmacological studies (Morales-Villagrán & Tapia 1996; Morales-Villagrán, et al., 1996), data that match well with the results showed here, although the main difference is that using biosensors for monitoring the brain the procedure can be done during a real time and with improved resolution. This work was supported by CONACyT project # 105 807.

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