# Is GABA excitatory or inhibitory at the AIS?

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# **1** Abstract

The fast-acting effects of the neurotransmitter y-aminobutyric acid (GABA) depend principally on the concentration gradient of chloride ions across the plasma membrane. In mature systems chloride levels are usually highest extracellularly and thus on activation of chloride-selective GABA<sub>A</sub> receptors, chloride ions flow into the cell causing a hyperpolarising and inhibitory response. Interestingly, in many areas of the cortex GABAergic interneurons selectively target the axon initial segment (AIS) in a mechanism classically thought to result in the inhibition of action potential initiation. However, recent evidence has suggested that this direct input to the AIS may in fact be excitatory due to a high chloride level within the structure. We decided to test this hypothesis in a simple *in vitro* primary culture system by uncaging CNB-caged GABA directly at GABAergic synapses on the AIS. We show that by targeting the uncaging laser at both the AIS and cell soma we can demonstrate GABA induced current responses. Despite this we observed a much larger GABA induced current from simply adding the 'caged' compound onto the cells suggesting that a large amount of GABA is not caged but is conversely active. Despite a sound experimental design, this inherent problem of the caged GABA compound unfortunately prevented us from completing this experiment.

# 2 Introduction

#### 2.1 Neuronal excitability

An individual neuron receives synaptic input from a large assortment of other neurons. These inputs are mediated by the binding of a pre-synaptically released neurotransmitter to the postsynaptic terminal of the synapse which directly or indirectly results in the flow of ions along their electrochemical gradient and across the plasma membrane. The neuron will fire an action potential if the sum of all of these synaptic inputs and thus flow of ions depolarises the cell to a threshold level. The integration of all this information and hence the 'decision' for action potential initiation occurs at the axon initial segment (AIS; Coombs *et al.*, 1957, Stuart and Hausser, 1994). This important region of the neuron, which separates somatodendritic and axonal compartments, is highly specialised for this role due to its ability to cluster high densities of voltage gated sodium channels (Kole *et al.*, 2008). Thus if the neuron reaches threshold, the sodium channels at the AIS will open, causing rapid sodium influx and resulting in an action potential being propagated both anterogradely along the axon and retrogradely back into the soma.

As a measure to prevent neuronal hyperexcitability and to further increase network computation, many of the synaptic inputs to neurons are inhibitory. These inputs

commonly make action potentials less likely to fire by taking the membrane potential further away from the threshold level in a process known as cell hyperpolarisation. The major neurotransmitter whose actions are inhibitory in the mature CNS is  $\gamma$ -aminobutyric acid (GABA). The binding of GABA to fast acting ionotropic GABA<sub>A</sub> receptors results in the opening of an ion channel which is selectively permeable to chloride ions. Therefore chloride, whose concentration is usually higher extracellularly, rushes along its electrochemical gradient making the cell interior more negative and resulting in hyperpolarisation. Additionally GABA can bind to metabotropic GABA<sub>B</sub> receptors, which act via secondary messenger cascades to open potassium channels also causing cell hyperpolarisation.

#### 2.2 GABAergic input to the AIS

*In vivo* many different types of GABAergic neurons are precisely targeted to different cellular compartment sto provide hyperpolarising and inhibitory input (Klausberger and Somogyi, 2008). Strikingly a number of specialised GABAergic interneurons in the cortex form synaptic connections exclusively with the AIS of glutamatergic pyramidal neurons (Figure 1). Somogyi and colleagues (1983) showed that the axon collaterals of a single axo-axonic cell in the monkey hippocampus can innervate over 200 pyramidal neurons at their AIS (Figure 1a). Moreover using immunoreactivity for the GABA<sub>A</sub>R scaffolding protein gephyrin, Cruz and colleagues (2009) show the specificity of GABAergic input at a single AIS; the puncta of gephyrin along the entire length of the AIS show individual GABAergic synapses (Figure 1b). These highly specific and targeted inputs to the AIS have been theorised to be hyperpolarising and to act as ideally positioned direct inhibitory modulation of action potential initiation.



Figure 1 - GABAergic interneurons interact with the axon initial segmentin vivo.

A) Camera Lucida drawing of a single Golgi stained axo-axonic cell of the CA1 region of the monkey hippocampus and its subsequent axon collateral interactions with the AIS of pyramidal cells. (a) axon, \* pyramidal cell bodies; Somogyi *et al.*, 1983. B) Brightfield photomicrograph of an AIS in area 46 of the primate prefrontal cortex immunoreactive for the GABA<sub>A</sub>R scaffolding protein gephyrin. P: pyramidal cell body, scale bar 5µm (Cruz *et al.*, 2009).

Despite the seemingly ideal positioning of GABAergic synapses on the AIS for inhibition, the actions of GABA directly depend on the balance of intracellular and extracellular chloride concentration. High levels of extracellular chloride cause it to rush into the cell upon GABA activation whereas in the opposite scenario, as seen early in development, high levels of intracellular chloride cause it to rush out of the cell resulting in cell depolarisation. In early development then, the actions of GABA are excitatory (reviewed by Ben-Ari, 2002). Indeed recent work has suggested that GABAergic inputs to the AIS are actually depolarising and excitatory, even in mature systems, due to a high level of chloride intracellularly at the AIS. Szabadics and colleagues (2006) showed that the reversal potential of GABA (EGABA) for inputs from GABAergic axo-axonic cells of layers 2/3 of rat somatosensory cortex was depolarising whereas the effects of GABAergic basket cells from the same layers which target the cell soma and proximal dendrites were hyperpolarising. They suggested that this effect was the result of a build up of chloride in the AIS due to a lack of the chloride efflux transporter KCC2. Furthermore, Khirug and colleagues (2008) used local photolysis of a caged GABA compound to measure E<sub>GABA</sub> at different cellular compartments. They showed that EGABA at the AIS was much more positive than in the somatodendritic compartment, therefore suggesting that the input was depolarising. This difference was abolished using the chloride influx transporter blocker NKCC1, again suggesting a high intracellular chloride concentration at the AIS. In contrast, a more recent paper by Glickfeld and colleagues (2009) stimulated individually at dendritic-targeting and AIS-targeting GABAergic neurons and subsequently measured unitary field potentials (ufield) near their pyramidal target cells. They showed that in both cases the ufield measured was the result of the hyperpolarising actions of synaptically released GABA and therefore argue that GABA is actually hyperpolarising at the AIS as classically thought.

Ultimately, it may not be too important whether the effects of GABA are depolarising or hyperpolarising at the AIS. Shunting inhibition is a mechanism activated when the reversal potential of a neurotransmitter is close to the resting potential of the cell. Therefore when the neurotransmitter binds and ion channels are opened there is a flow of ions but no large change in membrane potential. This flow of ions leads to an increase in the leak conductance of the membrane, so that larger inputs are needed to make the cell fire. Thus, even if neurotransmitter actions are slightly depolarising, they can still be inhibitory because they make action potential firing less likely. Subsequently it is more informative and thus more important to know if the GABAergic input at the AIS is excitatory or inhibitory rather than knowing whether it is depolarising or hyperpolarising.

#### 2.3 Project rationale

There is currently a large amount of controversy in this field and the issue as to whether GABAergic inputs at the AIS are excitatory or inhibitory remains to be resolved. We decided to address this question using a less complex *in vitro* culture system of dissociated hippocampal neurons, employing a caged GABA compound to activate GABAergic synapses directly and precisely at the AIS. We felt that our approach conveyed the following advantages:

#### - Live visualisation of the AIS.

Many proteins localise themselves to the AIS by binding to its AnkyrinG scaffold. Indeed the binding of voltage-gated sodium channels (Na<sub>v</sub>) is dependent upon a short amino acid sequence located intracellularly between their second and third transmembrane domain (Garrido*et al*,2003). The YFP-NavII-III construct was made by fusing the NavII-III loop directly to YFP and hence when expressed targets YFP specifically to the AIS (Grubb and Burrone, 2010; Figure 2a). This construct can therefore be used within live experiments to visualise the AIS. Previous studies uncaging GABA at the AIS have not shown its exact location and can therefore not be sure where they have uncaged (Khirug *et al.*, 2008, Katagiri*et al.*, 2007)

#### - Live visualisation of GABAergic synapses.

The Gephyrin-mcherry construct expresses the GABA<sub>A</sub>R scaffolding protein gephyrin fused to m-cherry (a gift from Corette Wierenga; Figure 2b; Maas *et al.,* 2006). This construct can therefore live label GABAergic synapses and coupled with a live AIS marker will allow us to visualise any of these synapses directly at the AIS, therefore showing exactly where we should uncage GABA. Again, previous experiments have not shown whether they have been uncaging directly at GABAergic synapses.

# - Ability to assess the effects on neuron excitability without affecting chloride concentration.

Previous studies looking at the actions of GABA at the AIS have used perforated patch clamping in electrophysiology experiments. This has the advantage of maintaining the concentration of intracellular chloride, however it also results in dialysis of the neuron with other components of the intracellular patch pipette solution and furthermore produces a mechanical disruption of the cell. To circumvent this problem we decided to stimulate the neuron directly with the patch pipette in an on-cell mode. This configuration seals onto the cell without rupturing the membrane and allows the detection of action potentials but not subthreshold events. We wanted to use the on-cell patch to firstly calculate the electrical current input needed to stimulate a single action potential to fire; then following uncaging of GABA at the AIS calculate this required input again. The read-out from this experiment would therefore give a direct assessment of whether the stimulus was excitatory or inhibitory; if the actions of GABA were

excitatory less electrical current input would be needed to stimulate an individual action potential, however if its actions were inhibitory we would need more electrical current input. Unfortunately stimulating the neuron using the patch pipette in on-cell mode proved difficult.

We therefore chose to switch our attention to stimulating the neurons by using the light activated cation channel channelrhodopsin-2 (ChR2). In dissociated hippocampal cultures photostimulation of ChR2 can mediate defined trains of action potential spikes with millisecond temporal resolution (Boyden et al., 2005). Therefore, instead of using electrical current as a stimulus we would determine how much blue light was needed to stimulate a single action potential to fire; thus if the actions of GABA were excitatory, less blue light would be required to stimulate an action potential to fire and conversely if GABA action was inhibitory, we would need more blue light to stimulate an action potential (Figure 3F). A drawback to using ChR2 (maximum: 460 nm; Nagel et al., 2003) in the same experiment as caged GABA (maximum: 260 nm) is that despite the separation of peak activation wavelengths, their activation spectra overlap; hence they can both activated by similar wavelengths of light. As a method of minimising this problem we acquired a ChR2 targeted specifically to the somatodendritic region of neurons (ChR2-MBD). This construct fuses channelrhodopsin to a myosin binding domain and subsequently when expressed it is restricted in its localisation to the somatodendritic region by myosin motors (a gift from D. Arnold, USC; Lewis et al., 2009, Figure 2c). The construct also contains a GFP, which in our experiment would interfere with the visualisation of live AIS marker. We consequently planned to exchange the GFP for a Myc tag.



**Figure 2 – Example images of constructs to be used in these experiments.** A) Confocal image of a dissociated hippocampal neuron expressing the YFP-NavII-III construct; white arrow indicates AIS position, white oval indicates cell soma. B) Example immunostaining of the colocalisation between the mRFP-gephyrin (red) construct expressed in hippocampal neurons and a gephyrin antibody (green; taken from Maas *et al.*, 2006). In our construct, the mRFP has been directly exchanged for m-cherry.C) L2/3 mouse cortical slices expressing ChR2 (left) and ChR2-MBD (right); blue: Dapi stain (taken from Lewis *et al.*, 2009).

Our final experimental design is depicted in figure 3. Dissociated hippocampal cultures are transfected at DIV7 with the YFP-NavII-III, gephyrin m-cherry and ChR2-MBD constructs. At DIV10 transfected cells are found on the confocal microscope and subjected to an on-cell patch. The neuron is then stimulated at the dendrites using a laser to activate ChR2 and the amount of laser required to induce a single action potential calculated. Finally GABA is uncaged directly at GABAergic synapses on the AIS and the neuron further stimulated by ChR2 to decipher the uncaging effect. We believed that this experimental design would give a direct indication of whether GABA and its actions at the AIS would make spiking more likely (GABA is excitatory) or less likely (GABA is inhibitory).



**Figure 3 – Schematic diagram of project Design.** At DIV7 dissociated hippocampal cultures are transfected with the live AIS marker (green box, A), live GABA synaptic marker (red circles, B) and ChR2-MBD (purple fill, C). At DIV10 neurons containing these constructs will be patch clamped in an on-cell configuration (D) before CNB caged GABA uncaging directly at GABAergic synapses on the AIS (E). The difference in laser power required to stimulate ChR2 and cause a single action potential to fire after GABA uncaging will subsequently be measured. The bar graph illustrates predicted results (F).

# 3 Materials and Methods

#### 3.1 Dissociated hippocampal neuron preparation

Dissociated hippocampal neurons were prepared from Sprague Dawley rat embryos dissected in HBSS at embryonic day 18. Hippocampi were digested with trypsin (Worthington, 0.5mg/ml; 15min 37°C) before trituration with rounded Pasteur pipettes of decreasing diameter. Dissociated cells were then plated in 24 well-plates (Corning) on 13mm coverslips pre-coated with poly-l-lysine at 50µg/ml (Sigma) and laminin at 40µg/ml at a density of 45,000 cells per well. Cells were cultured at 37°C with 6% CO<sub>2</sub> in 1ml total volume containing 500µl of Neurobasal medium supplemented with B27 (2%) plus Glutamax (500µM) and 500µl of Neurobasal medium supplemented with FCS (2%) plus Glutamax. After 3DIV and every 3days subsequently half of the media was replaced with Neurobasal containing 2% B27 and Glutamax. Cells were transfected at DIV7 with 1µg of DNA per well using lipofectamine 2000.

#### 3.2 Immunofluorescence

Dissociated hippocampal cells were fixed at appropriate time points by adding an equal volume of 4% PFA (final concentration 2% PFA) directly onto the culture medium for 5 minutes, followed by 4% PFA: PBS treatment for a further 20 minutes. Fixed cells were then washed into PBS for maintenance at 4°C until required.

For immunostaining, cells were permeabilised by washing in PBS: 0.25% Triton for 5 minutes followed by 1 hour of blocking with 3% bovine serum albumin (Sigma). After permeabilisation primary antibodies were added at appropriate concentrations in 1% BSA: PBS for 2 hours at room temperature or overnight at 4°C. Coverslips were then washed three times in PBSbefore the application of the appropriate secondary antibody for 45 minutes at room temperature. After three further washes with PBS, coverslips were mounted on glass slides using Mowiol (Calbiochem).

#### 3.3 Constructs

YFP-NavII-III was made by Dr Matthew Grubb to utilise the  $Na_v$  channel binding sequence to ankyrinG in order to target YFP to the AIS (Grubb and Burrone, 2010).

### 3.4 Caged GABA

CNB-caged GABA (Invitrogen) was made up in dim light using HBS (pH 7.4, ~280 mOsm (see below)) to a concentration of 25 mM (10x). The pH was then measured

using pH paper and increased using NaOH up to pH 7.4 before aliquoting and storage in the dark at -20°C. Aliquots were thawed immediately before an experiment on ice and used in a darkened room.

### 3.5 Electrophysiology

Whole-cell patch-clamp recordings were made from DIV10 dissociated hippocampal cultures in an extracellular solution (pH 7.4, ~280 mOsm, ~23 °C) containing in mM: 136 NaCl, 2.5 KCl, 10 HEPES, 10 D-glucose, 2 CaCl2, 1.3 MgCl2, 20 6-cyano-7nitroquinoxaline-2,3-dione (CNQX, Sigma, UK), 25 DL-2-amino-5-phosphonovaleric acid (APV, Sigma, UK). Patch pipettes were pulled using a P-97 Flaming/Brown type micropipette puller (Sutter instruments) from borosilicate glass (OD 1.5 mm, ID 1.17 mm, Harvard Apparatus, Edenbridge, UK), with a resistance of 3-6 M $\Omega$ , and were filled with an Cs-gluconate based internal solution containing, in mM: 130 Cs gluconate, 10 NaCl, 1 EGTA, 0.133 CaCl2, 2 MgCl2, 10 HEPES, 3.5 MgATP, 1 NaGTP. Recordings were acquired with a Multiclamp 700B amplifier coupled to pCLAMP acquisition software (Molecular Devices). Signals were Bessel filtered at 10 kHz, digitised, and sampled at 20 kHz (50 µs sample interval). Fast capacitance was compensated in the on-cell configuration. Upon a giga-ohm seal membranes were ruptured and cells were held at -40mV before increasing in +20mV increments to 0mV or +40mV voltage clamp where outward GABA events could be measured. Spontaneous protocols sampled for 30 seconds (600000 frames) whereas uncaging protocols sampled for 2 seconds (40000 frames). CNB-caged GABA (Invitrogen) was uncaged at designated areas of the cell using a near violet diode 405 nm laser for 500 ms at a strength of 100% (approx. 1mW at the coverslip surface). At the end of each recorded trace we used responses to a 10 ms, 10 mV depolarisation step to estimate the membrane resistance (Rm) of the neuron, from the steady holding current at the new voltage.

Analysis of data was performed using pCLAMP software. Both uncaging and CNBcaged GABA responses were calculated as the change in mean holding current from before to after the stimulus had been applied.

# 4 Results

#### 4.1 GABAergic synapses are present at the AIS in vitro

Firstly, in order to establish whether GABAergic synapses were present at the AIS our dissociated hippocampal culture system, we carried out immunostaining for AIS and synaptic markers. Co-labelling for both the AIS scaffolding protein ankyrinG and the pre-synaptic GABA synthesising enzyme GAD65 showed that many axon initial segments within the culture were opposed by GABAergic synapses (Figure 4a). The AIS was also shown to have excitatory inputs by immunostaining with Vglut1 (Figure 4b).



**Figure 4 – GABAergic and excitatory synapses are present on the AIS***in vitro***.** Double immunostaining of DIV10 dissociated hippocampal neurons for A) AnkyrinG and GAD65 and B) AnkyrinG and vGlut1. White arrows denote synapses on the AIS and the white oval indicates the cell body.

# 4.2 Testing Caged GABA

After proving that GABAergic synapses were present at the AIS in culture, we next decided to test the caged GABA compound to ascertain whether we could detect any GABA responses after an uncaging pulse from the 405 nm laser.

#### 4.2.1 Patch clamping

Before uncaging experiments could be implemented, I used the first month of the rotation learning and practising the patch clamping technique. This was performed on DIV10 hippocampal cultures; firstly pulling patch pipettes and finding their position on the microscope before moving the pipette down and positioning it in order to seal onto cells. During these experiments, I also learnt how to control the electrophysiology software to test pipette resistance and to compensate for both fast and slow capacitance. Finally, after learning to make a gigha-ohm seal on the

cells I also practised rupturing the membrane for whole-cell patch clamping. This technique was more difficult than that of on-cell patch clamping required for the final planned experiment, however it was necessary for measuring subthreshold GABA events. Indeed, in this mode we illustrated that at DIV10 the dissociated hippocampal cells showed spontaneous GABAergic events (Figure 5b).



#### Figure 5 – Whole cell patch clamping shows spontaneous GABAergic events.

A) Transmitted light image of a patch pipette sealed to a dissociated hippocampal cell in a whole-cell patch clamp recording. B) Spontaneous GABAergic events recorded whilst holding a cell at +40 mV in voltage-clamp mode.

#### 4.2.2 Effects of uncaging GABA

Once a competency in patch-clamping was attained we tested whether there was any uncaging response with the CNB-caged GABA compound at the cell soma and AIS. Hippocampal neurons transfected with the YFP NavII-III construct were transferred into HBS media containing the CNB-caged GABA compound at a concentration of 2.5mM (as used in Khirug *et al.*, 2009) for patch clamping. This extracellular medium also contained both an NMDA receptor blocker (APV) and an AMPA receptor blocker (NBQX) to silence all glutamatergic events. Furthermore, in order to reduce background and to ensure that the events observed were only due to chloride influx, we blocked the potassium channels within the recorded neuron using a caesium gluconate based internal pipette solution. Transfected neurons were located using epifluorescence before whole-cell patching; cells were initially held in voltage clamp at -40 mV before this was increased in +20 mV increments to a final holding potential of 0 mV. This holding voltage took the cell away from the reversal potential of GABA, thus producing detectable outward chloride currents upon GABAergic events.

GABA uncaging over selected areas including the cell soma and the AIS produced repeatable and reliable GABA-mediated current increases (n=3 cells). These were hard to compare quantitatively due to different cell morphologies and different holding currents; however all showed qualitatively similar small and slow GABA responses to the uncaging stimulus. Figure 6 shows recordings obtained from uncaging at different regions of a single cell. Uncaging GABA over the whole soma produced a GABA mediated current increase of 42.1 pA whereas uncaging over the

entire the AIS produced a current increase of 23.8 pA. On separation of the uncaging into proximal and distal AIS, the GABA mediated current was 17.7 pA and 17.1 pA respectively. These results illustrated that GABA could be uncaged to produce small but measureable responses at both the cell soma and at the AIS.



#### Figure 6 - GABA uncaging at the AIS causes a response in recorded hippocampal neurons.

<u>Top left</u>:Confocal image of a dissociated hippocampal neuron transfected with YFP-NavII-III taken with the 488 nm laser. <u>Left</u>: Transmitted light images of the cell in whole-cell patch clamp; regions of violet denote areas of GABA uncaging with 100% power of 405 nm laser. <u>Right</u>: recordings of uncaging-induced GABA mediated currents from the cell during and immediately after the uncaging stimuli; Violet bars indicate the length of the uncaging stimulus.

Despite the success in showing current responses after GABA uncaging, recordings during these experiments were very unstable. A very large amount of current ranging from 175pA-400pA (mean: 286.9 pA  $\pm$ 73.1 pA (S.E.M), n=3) had to be input through the electrode to maintain different cells at 0mV. Moreover the test pulse which steps the holding voltage 10mV and determines the physical relationship between the patch pipette and the cell (see methods) always illustrated a very low membrane resistance of between 10-30 MQ (mean: 18.9 MQ  $\pm$ 4.7 MQ (S.E.M), n= 3) and thus showed that the cell had a very 'leaky' membrane. In troubleshooting this problem we tested a large number of factors including changing internal solutions, testing the pH of all solutions and making up a new batch of caged GABA, but with no success. We eventually decided to test the CNB-caged GABA compound to distinguish whether this was causing the problem.

#### 4.2.3 CNB-caged GABA is not fully caged.

To test the CNB-caged GABA compound we decided to run patch clamp recordings of spontaneous activity in untransfected hippocampal cells minus the compound and then to run another recording whilst adding the compound. For this experiment, the compound was acquired and made up on the day of the experiment using the manufacturer's instructions; moreover after preparation it was maintained in the dark until use and added to the chamber during the experiment in a dark room. This experiment was repeated in two cells; figure 7 shows recordings obtained from a one of these cells. In the absence of CNB-caged GABA the current required to clamp the cell at 0mV was recorded at 21.4 pA, with an associated membrane resistance  $(R_m)$  of 176.1 M $\Omega$  (Figure 6, left). On addition of CNB-caged GABA to a final concentration of 2.5 mM (as used in Khirug et al., 2008) the current required to hold the cell at 0mV substantially increased to 654.9 pA, which consequently decreased the  $R_m$  to 8.9 M $\Omega$ (Figure 6, middle left). This result was highly suggestive of a constant GABAergic current and thus to conclusively prove that this effect was due to the activity of the GABA compound we performed further recordings whilst adding both GABA<sub>A</sub> and GABA<sub>B</sub> receptor antagonists. Indeed addition of  $10\mu$ M and later 100µM of the GABA<sub>A</sub> receptor antagonist SR-95531 (Gabazine) decreased this response to 91.7% and 66% of its peak respectively and increased  $R_m$  to 12.8 M $\Omega$ (Figure 6, middle right). Furthermore, addition of 50µM GABA<sub>B</sub> receptor antagonist CGP 55845 almost totally abolished the GABA induced current, bringing the input current down to 27.5pA and thus 0.96% of the total response; R<sub>m</sub> was increased to 202.4 M $\Omega$  (Figure 6, right).



**Figure 7–Caged GABA is not fully caged.** Whole cell patch-clamp (top) and test pulse (bottom) recordings of dissociated hippocampal cultures treated with CNB-caged GABA. A selected neuron was subjected to voltage clamp at 0mV and spontaneous events recorded (Left) before addition of CNB-caged GABA (2.5mM; middle left). The GABA mediated current increase was then blocked sequentially by addition of GABA<sub>A</sub> antagonist SR-95531 and GABA<sub>B</sub> antagonist CGP 55845.

Interestingly Katagiri and colleagues(2007) used the caged GABA compound at a much lower concentration (10 $\mu$ M) and observed easily measureable GABA evoked responses in acute slices of visual cortex in mouse. We therefore performed the same experiment again, firstly clamping the cell at +40 mV to increase the sensitivity of the recording to outward GABAergic events, before measuring spontaneous events and finally adding the CNB-caged GABA (Figure 7A). Addition of the caged GABA compound at this lower concentration resulted in a much smaller response than before but still increased the input current by 40.3 pA and dramatically increased the number of spontaneous events that could be seen. We then attempted to uncage GABA at a large area directly over the cell body but failed even with a long laser stimulus to see any GABA mediated current responses at this concentration (Figure 8B).



Figure 8–Chronic GABA mediated current responses can still be seen at a lower concentration of CNB-caged GABA. Whole cell patch-clamp recordings of dissociated hippocampal cultures treated with CNB-caged GABA. A) A selected neuron was subjected to voltage clamp at +40 mV and spontaneous events recorded (Left) before addition of CNB-caged GABA (10  $\mu$ M; right). Boxes magnify regions of interest. B) Recordings of cell after uncaging GABA at the cell soma. Violet box denotes the 500 ms of uncaging with the 405 nm laser at 100% strength.

# **5** Discussion

Our experiment was designed to activate the CNB-caged GABA compound directly at GABAergic synapses of the AIS in an attempt to determine the function of GABA at these synapses. We firstly showed that these synapses exist in the *in vitro* dissociated hippocampal system and that we could evoke measurable GABA induced currents at both the AIS and cell soma by uncaging. However further testing of the caged GABA compound showed that the GABA was largely active despite not being physically activated using the UV laser.

#### 5.1 Why do we see such large chronic GABA-mediated currents?

The results obtained from our experiments showed that the caged GABA compound is active even without UV stimulation; a single patch-clamped cell showed a large GABA mediated current increase of 654.9 pA when the compound was added at a concentration of 2.5mM. This effect was not described by Khirug and colleagues who used the same compound at this concentration. They performed uncaging in hippocampal slice cultures, a technique which involves culturing pieces of tissue and has the advantage of maintaining much of the cellular organisation. It is possible that only a much lower concentration of the GABA compound was reaching and acting upon the target cells due to GABA uptake mechanisms by other cells within the tissue (Conti et al., 2004), whereas in our dissociated and culture system there would be less cellular compensation and the GABA compound would be directly acting upon the cells. Interestingly, on using much lower concentrations (10µM) as described by Katagiri and colleagues (2007) we observed a much lower current increase on CNB-caged GABA addition, although in slice recordings this effect was never seen (H. Katagiri, personal communication). It is therefore possible that we did not protect the caged GABA compound enough from light sources during our preparation. It is also important to consider that the tissue in slice preparations might determine the effects of caged GABA. However given the huge chronic currents we observed, the reliability of experimental results using such high concentrations of the caged compound could be questioned.

During our experiments, we also observed GABA-mediated current increases upon uncaging. However despite GABA activation over large areas of the soma and AIS the responses measured were much smaller than those reported within the other papers discussed (Khirug *et al.,* 2008 and Katagiri*et al.,* 2007). Firstly, it is possible that part of this effect was brought about by the activity of the CNB-caged GABA compound. The pre-active GABA could have been occupying a large proportion of GABA receptors and thus any GABA increase caused by uncaging could only activate the small number of free receptors remaining, resulting in only a small current increase. Moreover the high background noise created by the observed leak currents could have been masking a small part of the response. Secondly, it is important to remember that the number of synapses at the AIS *in vitro* (Figure 4a) is much lower than the number of synapse at the AIS *in vivo* (Figure 1b). This factor would be likely to make the change in current observed through whole-cell patching smaller after uncaging *in vitro*. Finally, the peak activation wavelength of CNB-caged GABA is 260 nm; in our experiments we were using a 405 nm laser. Although this was activating the caged compound, it may be that by using a laser closer to 260 nm we would have seen bigger GABA responses.

#### 5.2 Future Directions

Before performing this experiment again it will be important to acquire a version of the caged GABA compound that does not show such non-caged GABA mediated responses. Recently a new version of caged GABA (RuBi GABA) compound has been synthesised; this compound uses a Ruthenium complex as a photosensor, which has faster uncaging kinetics and can be activated by visible light. It has also been shown to produce GABA-mediated currents *in vitro* in pyramidal neurons (Rial Verde *et al.,* 2008). It would be interesting to test this caged compound on our *in vitro* culture system to see if we saw GABA-mediated currents before attempting uncaging.

When cells are transfected with the ChR2-MBD construct we risk uncaging whilst activating ChR2 due to their overlapping excitation spectra. Therefore the experiment would be improved by using a ChR2 protein activated by different wavelength of light. Zhang and colleagues (2008) recently described Volvox Channelrhodopsin (VChR1), a similar light activated cation channel from the algae *Volvox carteri* with excitation maxima red-shifted 70nm. The expression and excitation of this construct should therefore not effect GABA uncaging.

Finally, if the experimental design described above was successful we could further consolidate our results by testing a similar setup in slice cultures. This would give the added advantage of maintaining cellular organistation whilst still allowing successful transfection with both live AIS and GABA synapse markers. We could then use defined fibre bundles of afferent neurons to stimulate a transfected cell and measure the current needed to induce the cell to fire a single action potential; subsequent repetition of this stimulation after uncaging would allow us to deduce the function of GABA at the AIS. This design would therefore also remove the need for transfection with ChR2.

It will be essential to eventually decipher the actions of GABA at the AIS in order to fully understand the role that GABAergic interneurons play in cortical microcircuits.

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