

Investigating neural communication with scanning ion conductance microscopy

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Abstract

The microelectrode pipette used for imaging by **scanning ion conductance microscopy** may also be applied for cell-attached recording from the fine structures imaged. This combination of techniques can potentially determine the localisation of ion channels within particular cellular regions. I aim to use this approach to investigate the mechanism of a form of synaptic plasticity in the cerebellum, **depolarisation-induced potentiation of inhibition**. This is believed to be mediated by a retrograde messenger acting on presynaptic NMDA receptors. However, the presence of functional NMDA receptors in the relevant presynaptic terminals is disputed. My intention is to resolve this question by recording directly from identified presynaptic boutons.

1 Introduction

Synapses from molecular-layer interneurons onto Purkinje cells in the cerebellum exhibit at least three distinct forms of plasticity driven by post-synaptic depolarisation: *rebound potentiation* (RP) (Llano et al. 1991a; Kano et al. 1992), *depolarisation-induced suppression of inhibition* (DSI) (Pitler and Alger 1992; Vincent et al. 1992; Marty and Llano 1995) and *depolarisation-induced potentiation of inhibition* (DPI) (Duguid and Smart 2004). While RP acts post-synaptically to increase the sensitivity of response, DSI and DPI both act presynaptically to modulate release probability. These presynaptic effects of post-synaptic activity imply the involvement of retrograde signalling pathways.

In the case of DSI, the retrograde messenger is the endocannabinoid 2-arachidonoylglycerol (Szabo et al. 2006), released from the Purkinje cell dendrites in response to elevated $[Ca^{2+}]_i$ (Diana et al. 2002; Kreitzer et al. 2002; Yoshida et al. 2002; Brenowitz and Regehr 2003). The signal acts on presynaptic CB1 receptors and leads to reduced transmission both by inhibiting calcium influx through voltage-gated calcium channels (VGCCs) (Diana et al. 2002) and by inducing a small shift in the whole cell potassium conductance that in turn reduces excitability (Kreitzer et al. 2002).

For DPI, the messenger is believed to be glutamate or a glutamate analogue (Duguid and Smart 2004, 2008). As with the endocannabinoid, release is triggered by a sharp increase in dendritic $[Ca^{2+}]_i$. It can be abolished by dialysing the Purkinje cell with botulinum toxin B, suggesting that the release mechanism is SNARE-dependent vesicular fusion (Duguid et al. 2007; Shin et al. 2008). In addition to causing DPI, the released glutamate can activate mGluR1 receptors on the Purkinje cell itself (Duguid et al. 2007; Shin et al. 2008), and may also have presynaptic effects at excitatory parallel fibre-Purkinje cell synapses (Levenes et al. 2001).

DPI can be simulated by pressure application of N-methyl-D-aspartate (NMDA). Moreover, it is abolished by the selective NMDA receptor (NMDAR) antagonist D-2-amino-5-phosphonopentanoate (AP5), strongly indicating a role for NMDARs in transducing the glutamate signal (Duguid and Smart 2004).

The increase in release probability that defines DPI depends on elevation of $[Ca^{2+}]_i$ in the presynaptic terminal. NMDARs exhibit a significant calcium permeability (Ascher and Nowak 1988; Hille 2001), so their activation would be expected to lead to some influx. Indeed, this can trigger vesicle release (Glitsch 2008), but is not sufficient in itself to account for a sustained $[Ca^{2+}]_i$ rise on the time scale of DPI. However, DPI can also be abolished by using ryanodine to block calcium release from internal stores. This suggests a dependence on calcium-induced calcium release (CICR), which could amplify the initial NMDAR calcium influx and produce the necessary elevation (Duguid and Smart 2004).

Appealing as this mechanism is, it requires that NMDARs be present on interneuron terminals. NMDAR subunits have been shown at this location in cultured cells by immunostaining (Duguid and Smart 2004), but this does not prove there are functional receptors and the matter remains controversial. A recent paper by Christie and Jahr (2008) argues that functional NMDARs are present only on the soma and dendrites of cerebellar interneurons,¹ and that effects on axonal $[Ca^{2+}]_i$ are instead produced by VGCCs activated by a passively-propagated depolarisation. This conclusion, based largely on calcium imaging, is difficult to square with the evidence for DPI: somatodendritic NMDAR activation does not offer a persuasive explanation of the phenomenon. In particular, DPI has been demonstrated in dissociated Purkinje

¹The authors only present data from stellate cells, but mention in passing that they have seen the same results from basket cells in as-yet unpublished experiments.

cells with only the terminals of the interneurons present (Duguid et al. 2007). Moreover, the researchers' use of cyclopiazonic acid (CPA) to suppress CICR means they may be blocking the very mechanism by which DPI is hypothesised to operate. Nevertheless, their results pose a problem for the localised NMDAR model of DPI action, and further evidence is needed before the matter can be decided.

Scanning ion conductance microscopy (SICM) is a scanning probe microscopy (SPM) able to produce high-resolution topographic images in physiologically-relevant conditions (Hansma et al. 1989; Korchev et al. 1997a, b). A glass microelectrode pipette is used as a proximity detector. When the probe is immersed in electrolyte and a voltage applied between its internal wire and a ground electrode in the bath, a current flows in the form of ions. On approaching the less-conductive surface of a sample such as a living cell, the ion flow is occluded, leading to a measurable drop in current. This drop is sensitive to very small changes in proximity, allowing the surface position to be determined with high precision. Piezo-electric actuators are used to accurately control the relative position of the sample and probe. By scanning the probe across the sample and collecting height measurements at many points, a map of the surface is obtained.

Because SICM detects a current drop while still some distance away from the surface, the technique is contact-free. It should therefore be ideal for imaging soft, fragile samples such as neurons, whose structure would be easily disrupted. However, the probe's region of sensitivity is highly localised at the tip, with very little capacity for detection to the sides. The traditional scanning mode is therefore only suited to fairly flat surfaces with gradual changes in height.

An alternative approach, termed 'hopping probe' mode, has recently been developed by our collaborators at Imperial College (Novak et al. 2009). In this mode, instead of scanning across the sample with continuous feedback, each surface pixel is taken as an independent measurement, with the probe withdrawn in between. Lateral movements occur with the probe far enough away from the surface to avoid collisions, and far more convoluted samples may be readily imaged. For the first time, this means complex neuronal networks can be scanned with a reasonable chance of success.

The SICM microelectrode is very similar to those used for traditional patch clamp recording (Sakmann and Neher 1995), albeit usually of higher resistance. The piezo actuators allow for very precise positioning, and a SICM image itself constitutes a detailed positional map measured within the piezos' reference frame. Using the image as a guide, the probe may readily be manoeuvred to target fine structures on the cell surface and perform cell-attached recordings there (Gorelik et al. 2002). Such 'smart' patch clamping can provide detailed information about the localisation of ion channels to particular regions of a cell (Gu et al. 2002).

These techniques are still under development and not in widespread use. By combining the hopping mode's ability to image complex neuronal networks with the localised electrophysiology of smart patch clamping, we aim to investigate whether or not functional NMDARs exist on the presynaptic terminals of cerebellar molecular layer interneurons and thereby elucidate the mechanism of DPI.

As is inevitable with cutting edge techniques, putting them into practice is not a simple matter of off-the-shelf equipment and straightforward, well-documented implementation. Many of the problems encountered when setting up are previously unknown, or have solutions that only apply in different, often irrelevant, circumstances. In consequence, much of the work I have done to date has been dedicated to getting the technology working to the point where it could be applicable to address biological questions.

2 Methods

2.1 Cell culture

Hippocampal cultures were prepared (separately) from P4 Sprague-Dawley rats and from P7 GAD65-GFP transgenic mice, as follows. The animal was killed by cervical dislocation and then decapitated. The brain was swiftly removed into ice cold dissection medium,² hemisected and sliced into 500 μ m coronal sections with a MacIlwain tissue chopper. The hippocampal CA1 and CA3 regions were dissected from the slices and incubated for 1 hour in trypsin solution, replacing the solution after 30 min. The tissue was then washed in BSA solution and triturated with fire-polished glass Pasteur pipettes. The supernatant was centrifuged at $110\times g$ for 5 min, and the cells resuspended in neurobasal medium with newborn calf serum (NCS). This suspension was plated onto thickness 0 glass coverslips, which had previously been coated with poly-D-lysine and washed in NCS for the final 30 min. After incubating overnight, the medium was replaced with neurobasal lacking NCS and the cells incubated for a further 3-14 days before use.

Cerebellar cultures were prepared from P7 GAD65-GFP transgenic mice as follows. The animal was killed by cervical dislocation and then decapitated. The cerebellum was swiftly removed into ice cold dissection medium and extraneous tissue and meninges removed. The cerebellar tissue was cut into small fragments and incubated for 25 min in trypsin, then washed in growth medium and triturated with a fire-polished glass Pasteur pipette. Undissociated tissue was allowed to settle out at room temperature. The suspension was centrifuged at $500\times g$ for 5 min at 4°C and the cell pellet resuspended in growth medium. The suspension was plated in small quantities (~ 0.3 ml) onto thickness 0 glass coverslips coated with laminin and incubated overnight. The dishes were then flooded with growth medium to ~ 2 ml and the cultures incubated for another 7-14 days before use.

Cells were incubated at 37°C in 95% O₂/5% CO₂.

2.2 Vibrodissociation

Cells were mechanically dissociated from acute slices of P10 Sprague-Dawley rat cerebellum using a modified 'vibrating ball' technique (Vorobjev 1991; Duguid et al. 2007), as follows. The animal was killed by cervical dislocation and then decapitated. The cerebellum was swiftly removed into ice cold VD cutting solution, and sliced into 500 μ m parasagittal sections using a MacIlwain tissue chopper. The slices were transferred to a chamber containing slice incubation solution and bubbled with 95% O₂/5% CO₂ for at least 1 hour at room temperature.

To prepare for experimentation, one slice was removed into a 35 mm petri dish containing vibrodissociation solution, and held in place using a platinum wire 'harp' threaded with fine nylon strands. Dissociation was performed using a glass rod bent into an L shape, one end of which had been melted into a smooth ball of ~ 1 mm diameter, while the other was glued to the cone of a 2.5" 64 Ω miniature loudspeaker (Farnell). The ball end was placed in the bath solution close to the slice surface and moved slowly back and forth over the Purkinje layer region of the slice, while a square pulse of approximately 500 Hz frequency and 10V amplitude was fed to the loudspeaker from a Grass S48 physiological stimulator. After 1-2 min of stimulation, the slice was removed and the dissociated cells allowed to settle for 15 min before use.

²For the composition of this and subsequent solutions see §2.6

2.3 Fluorescence microscopy

Cells were loaded with styryl dye—FM 1-43 or SynaptoRed—using either stimulated or spontaneous activity. In the former case, a high $[K^+]$ FM loading solution was used to depolarise the cells, in the latter our standard recording solution. In either case, a 10 mM stock solution of dye was added in the proportion 1:1000 (v/v), for a final dye concentration of 10 μ M. This resulting solution was applied to the cells for 1 min, and then washed off 4-5 times in normal recording solution, with 4 min between washes. Cells were kept in darkness during the loading and wash-out periods.³

Cultures prepared from GAD65-GFP mice expressed GFP in a proportion of inhibitory interneurons without further intervention.

Optical microscopy was performed on a Nikon Eclipse TE-2000U inverted microscope fitted with the following Nikon objectives: Ph1 ADL 10 $\times$ /0.25 NA; Plan Fluor 60 $\times$ /0.85 NA; Plan 100 $\times$ /1.25 NA oil immersion. Fluorescence was excited with a Nikon Intensilight C-HGFI source. A Semrock Brightline FITC filter set (465-495/505/515-555) was used to image GFP and FM 1-43. SynaptoRed was imaged with a Chroma Technology HQ545/30 $\times$ excitation filter and Q570LP dichroic, together with a Semrock EdgeBasic 580nm long pass emission filter. As described in §3.4, brightfield illumination in general occurred without phase rings.

Images were captured from a Watec WAT-120N camera via a MatrixVision mvDelta frame grabber board, using DirectShow drivers and VLC software (<http://www.videolan.org/vlc/>). Images were viewed and processed using Picture Viewer (Microsoft) and Photoshop (Adobe Systems).

2.4 Scanning ion conductance microscopy

The scanning ion conductance equipment was supplied by IonScope Ltd and controlled by their ScanIC Control software. Stage positioning DC motors, piezo actuators and high-voltage amplifiers were by Physike Instrumente. The SICM and optical microscope were mounted on a Halcyonics Active Workstation 900 anti-vibration table and placed inside a custom-built Faraday cage.

Command voltages were generated and monitored using an Axopatch 200B patch clamp amplifier and Clampex 10 software, communicating via a Digidata 1440A interface (all Axon Instruments).

Pipettes were pulled from 75 mm long 1.0 mm OD 0.58 mm ID filament borosilicate glass capillaries with internal filaments (Intracel) using a Sutter Instruments P-2000 laser puller with the following settings:

HEAT=350 FIL=3 VEL=30 DEL=200 PUL=
HEAT=250 FIL=2 VEL=27 DEL=160 PUL=250

Pipette resistance, measured using the Clampex membrane test pulse with standard recording solution in both the pipette and bath, was typically 80-120 M Ω ; baseline current was accordingly $\sim$ 2 nA for a command voltage of 200 mV, with RMS noise 1.0-1.3 pA (as reported by the amplifier). Except during some specific tests of scanning with other pipette parameters, pipettes of significantly different resistance or exhibiting greater noise levels were discarded.

³In some cases, the glutamate receptor blockers DNQX and AP5 were added to the loading and wash-out solutions at 2 μ M each, in an attempt to reduce any excitotoxic effects of loading. However, this did not affect any of the results presented here.

As discussed in §3.3, pipettes were initially untreated, but in later experiments were coated with Sylgard elastomer (Dow Corning).

The current signal was filtered at 2 kHz using the amplifier's internal 4-pole Bessel filter and delivered to the SICM digital signal processor (DSP) at a gain of 2 mV/pA, with the amplifier operating in 'whole cell' mode. The SICM DC break setpoint was adjusted according to the noise level and profile of the current signal, ranging in most cases from $70\text{--}80 \times 10^{-4}$ V, equivalent to a current drop of ~ 4 pA or $\sim 0.2\%$ of baseline. This was typically 3-4 times the RMS noise.

Images were viewed and processed using the open-source SPM analysis application Gwyddion (<http://gwyddion.net/>). Code to allow Gwyddion to read SICM data files was written and contributed to the project.

2.5 Electrophysiology

Cell-attached patch recordings were made using the same apparatus as for SICM, described above. Using a prior scan as reference, the pipette was moved to a location of interest in the X-Y plane and stopped several μm above the measured surface height at that point. SICM feedback control of the pipette was suspended, and a membrane test pulse of -20 mV (from a holding voltage of 0 mV) applied using Clampex.

The pipette was lowered by direct manual command in small (10-100nm) increments until a slight resistance increase was observed. The pipette potential was then adjusted to between -40 and -60 mV and light suction applied by mouth to initiate a seal. If a stable seal of at least $10\text{ G}\Omega$ was obtained, the amplifier was switched to 'patch' mode, the output gain increased to $50\text{--}100\times$, and recording commenced. Pipette potential was adjusted manually during recording to obtain I-V dependency data.

All recordings discussed here were made using standard recording solution both in the bath and the pipette.

Recordings were viewed and analysed using Clampfit (Axon Instruments), Igor Pro (WaveMetrics) and R (<http://www.r-project.org/>).

2.6 Solutions

Dissection medium	Gey's balanced salt solution with additional MgCl ₂ 8 mM and D-glucose 33 mM
Trypsin solution	Trypsin (type XI T-1005) 1 mg/ml in HBSS with HEPES 8 mM
BSA solution	Bovine albumin 1 mg/ml in HBSS 90% and NCS 10% v/v with additional MgCl ₂ 8 mM
Neurobasal without NCS	(v/v) Neurobasal medium 97.9%, L-glutamine (100×) 0.2%, B27 (50×) 1.9%
Neurobasal with NCS	(v/v) Neurobasal medium 93.6%, L-glutamine (100×) 0.2%, B27 (50×) 1.8%, NCS 4.4%
Cerebellar growth medium	(v/v) Neurobasal-A 95%, N2 (100×) 1%, B27 (50×) 2%, L-glutamine (100×) 1% and P/S 1%
Vibrodissociation solution	(mM) NaCl 145, KCl 3, D-glucose 15, HEPES 10, MgCl ₂ 3, CaCl ₂ 0.5 — pH adjusted to 7.35 with NaOH
VD cutting solution	(mM) NaCl 135, KCl 3, NaHCO ₃ 20, NaH ₂ PO ₄ 1, D-glucose 15, MgCl ₂ 3, CaCl ₂ 0.5 — bubbled with 95% O ₂ /5% CO ₂
Slice incubation solution	(mM) NaCl 135, KCl 3, NaHCO ₃ 20, NaH ₂ PO ₄ 1, D-glucose 15, MgCl ₂ 1, CaCl ₂ 25 — bubbled with 95% O ₂ /5% CO ₂
Recording solution	(mM) NaCl 137, KCl 3, D-glucose 11, HEPES 10, MgCl ₂ 1.5, CaCl ₂ 2.5 — pH adjusted to 7.2 with NaOH
FM loading solution	(mM) NaCl 100, KCl 40, D-glucose 11, HEPES 10, MgCl ₂ 1.5, CaCl ₂ 2.5 — pH adjusted to 7.2 with NaOH

All solutions were filtered with Nalgene 0.2 μ m filters before use.

Gey's, trypsin, AP5, DNQX, MgCl₂ solution and sterile water were obtained from Sigma. HBSS, Neurobasal medium, Neurobasal-A, NCS, P/S, laminin, N2, B27, L-glutamine, poly-D-lysine and FM 1-43 were supplied by Invitrogen. SynaptoRed (FM 4-64) was from Merck. Bovine albumin was from ICN Biochemicals. All other reagents were supplied by VWR.

3 Results

3.1 Factors affecting the viability of SICM

By its nature, scanning probe imaging requires accurate positioning, consistency of measurement, and electrical and mechanical stability of both equipment and sample throughout the scan. It is susceptible to disruption by a number of factors that affect the signal fidelity or positional stability.

A key component of the scan equipment is the probe itself. Like a patch pipette, this is single-use and must be pulled afresh for each experiment. While the P-2000 laser puller is designed to maximise reproducibility of the parameters affecting the pull, there is still some variability in the resulting pipettes.

The pipette tip geometry cannot easily be directly measured, but it affects the resistance and the way the conductance varies on approach to the sample surface. The former is routinely monitored for every pipette and those outside the correct range discarded, but the latter is not explicitly determined. This is an issue because incorrect approach characteristics may confound or prevent scanning.

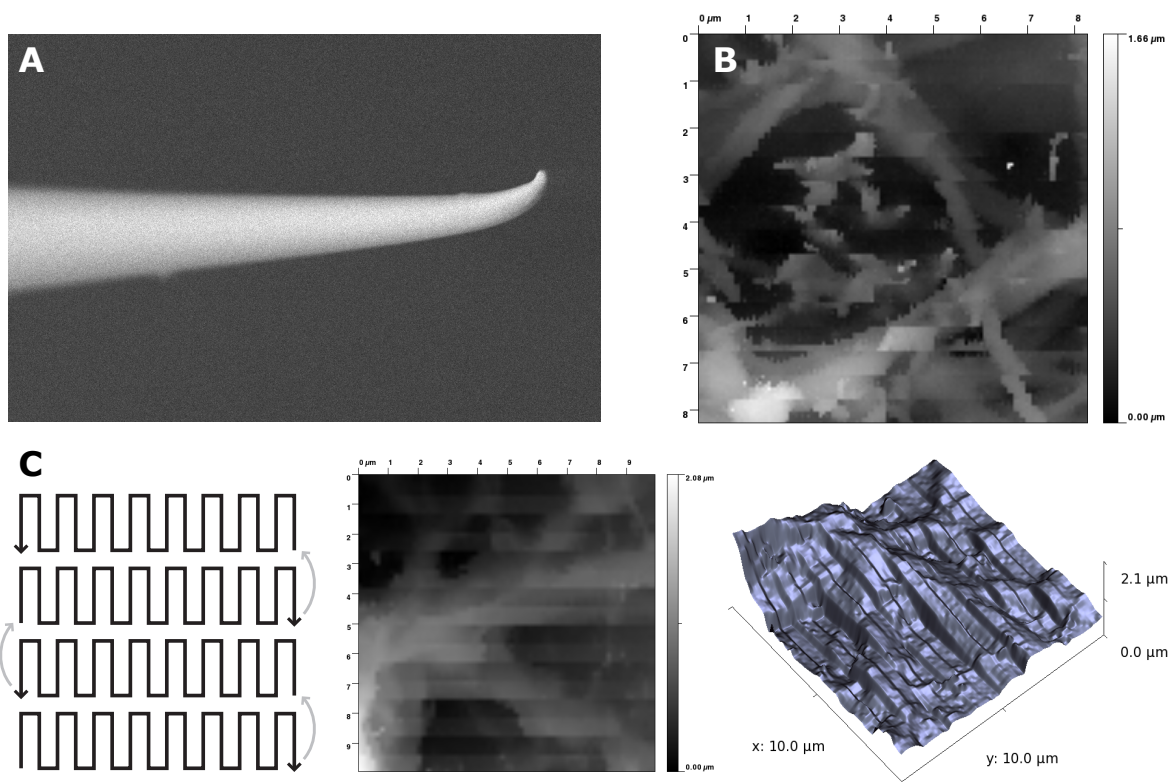


Figure 1 Scanning is prone to disruption from numerous sources.

A Scanning electron micrograph of a pipette tip with pronounced asymmetry. Such tips cause problems for SICM imaging.

B SICM scan of a network of processes in hippocampal culture. In this and subsequent SICM images, grey levels indicate the height measured at each point; the false colour bar to the right indicates the vertical scale. Even small movements or drifting in the sample over the duration of a scan manifest as discontinuities like those seen here. One notable source of such motion is perfusion.

C The 'boustrophedon' sequence used for sampling in hopping mode (left) can translate many underlying issues of positional stability into seemingly-systematic stripe artefacts. Shown centre and right are two different representations of a hippocampal culture scan exhibiting such striping: a flat greyscale view as before, and a 3D rendering of the same data.

Initial attempts to get SICM working were unsuccessful. In particular, the pipette very often crashed into the sample surface. Examination of a number of pipette tips using scanning electron microscopy (SEM) revealed that some were profoundly asymmetrical, as in figure 1A. In such cases, the region of sensitivity at the pipette tip will not coincide sufficiently with the direction of approach, leading to the observed crashes.

This asymmetry may result from uneven heating in the puller, caused by drift in alignment of the laser and an accretion of dirt on its parabolic mirror. Regular cleaning and realignment substantially reduces the number of crashes.

SICM is quite slow in comparison with most optical techniques. Even small, low-resolution scans take over a minute, while more detailed images, like the majority of those shown in this report, take 8-10 minutes or even longer. This is a long time to maintain positional consistency, especially in live samples, and in general some degree of variation is unavoidable.

Positional changes during the course of a scan give rise to artefacts and distortions in the final image, as seen for example in figure 1B. A number of different kinds of error may appear,

depending on the nature of the movement in the sample or equipment. Most commonly these will manifest as stripes or lines of discontinuity between regions imaged at different times. This is a consequence of the scan pattern used in the hopping probe protocol, a bidirectional ‘boustrophedon’ sequence illustrated in the left panel of figure 1C. The centre and right panels show an example of the resulting stripes.

One major source of motion disturbance is bath perfusion, and in fact this seems to be incompatible with successful scanning. Ideally, one would like to maintain perfusion between scans, only switching it off while actually performing a scan. However, we have not yet managed to get this to work consistently and need to refine the perfusion set-up. At present most experiments are performed with no perfusion at all. This is somewhat limiting in terms of the interventions that can be made, and more importantly seems to have an adverse effect on cell health.

Another common cause of errors is drift due to thermal expansion and contraction of the apparatus. Even quite small temperature changes ($\sim 0.5^\circ\text{C}$ over the course of several minutes) can lead to movements of several μm in the stage, enough to cause significant disruption of the scan. Therefore, as far as possible, all local sources of heating, cooling and air movement are now switched off while using the SICM. We have also taken to shrouding the microscope with a cloth to protect it from draughts. The biggest remaining driver of temperature change is the overhead brightfield lamp. The illumination this provides is in any case unsatisfactory, as discussed below (§3.4), and we are looking at replacing it with some other source that is cold, remote or both. At present, though, the lamp is all we have; and is usually workable provided the system is given plenty of time to equilibrate before scanning.

3.2 Surface imaging requires ‘clean’ preparations

SICM, like any topographic imaging technique, is only able to measure the uppermost surface of a sample. For this to be useful, that surface needs to include the elements of interest. This is not always so for neuronal preparations.

Perhaps the most popular preparation for studying synaptic activity is the acute brain slice (Kerkut and Wheal 1981; Dingledine 1984). Such a slice preserves most of the morphology and connectivity of the corresponding brain region *in vivo*, and may also preserve much of the local function. These details can be observed using optical microscopy, which is able to penetrate some way beneath the cut surface, and can be recorded electrophysiologically by burrowing the patch pipette down to the cells of interest using strong positive pressure to nudge unwanted material out of the way.

The process of slicing, however, exposes a surface consisting largely of debris and severed tissue. It is this damaged layer that SICM reveals, as seen in figure 2A. While this might be of interest for some purposes, it does not provide a useful context for selecting healthy, connected cells for patch recording.

An alternative is to use the *nerve-bouton preparation* (Vorobjev 1991; Akaike and Moorhouse 2003), produced by mechanically dissociating cells from an acute slice by means of acoustic vibration. This reductionist approach has the benefit of releasing healthy cells from their surroundings, together with functioning synapses. The presynaptic boutons in such cases have lost their axonal connection to their own neuron and instead remain attached to their postsynaptic partner. These boutons continue to function for a considerable time in this detached state; although it is certainly possible to argue that their behaviour might not exactly conform to that of *in vivo* counterparts that have not been subjected to such an assault.

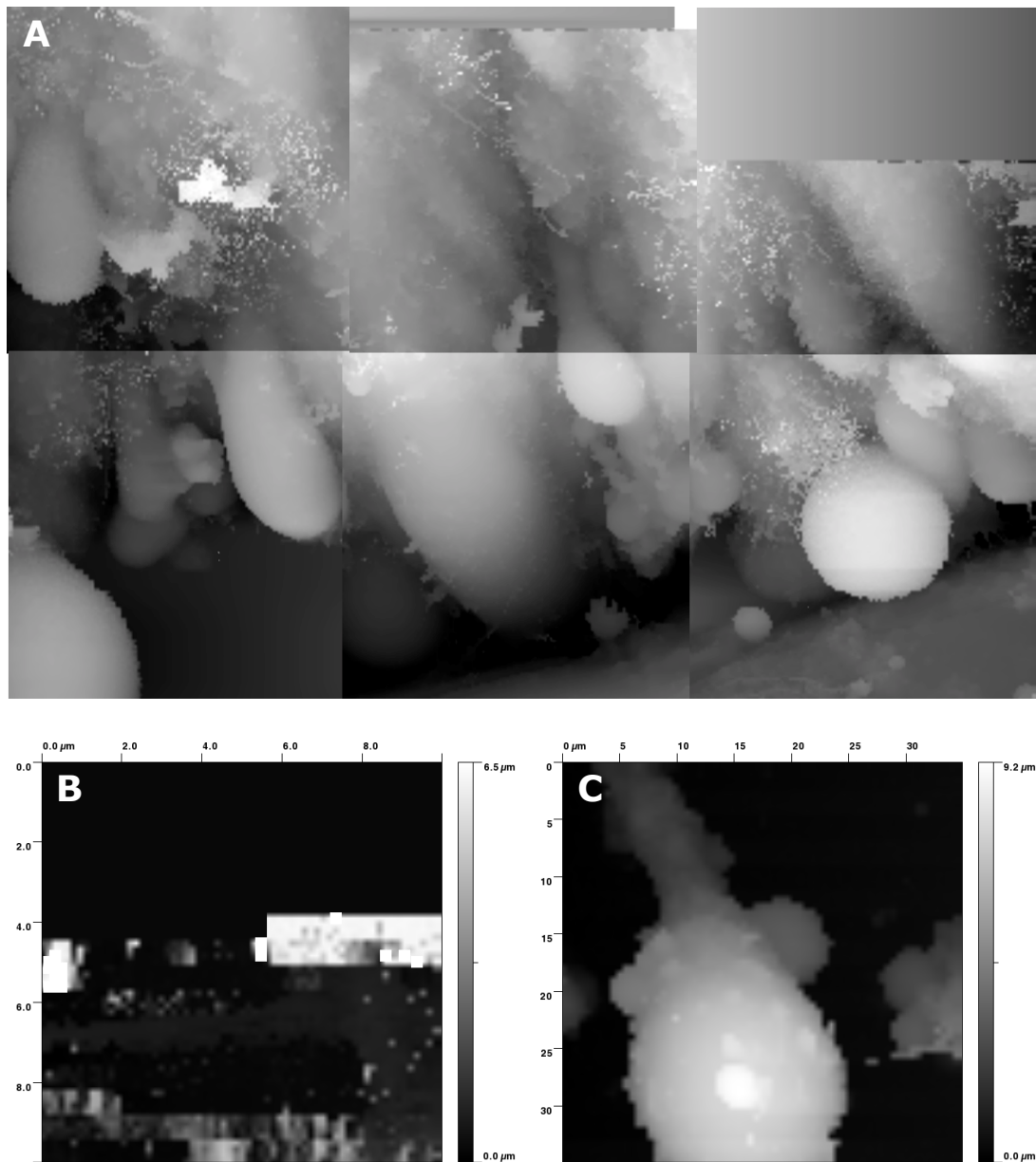


Figure 2 Topographic imaging is problematic for preparations with a 'dirty' surface.

A Composite scan of part of an acute slice of rat hippocampus. The overall vertical span was greater than the 25 μm range of the Z piezo, so the region had to be scanned in separate tiles that were then pieced together manually. The accessible surface was largely debris and cut cells, obscuring any intact morphology beneath. (*Image courtesy of Yuri Korchev.*)

B Typical scan of a vibrodissociated neuron. The combination of debris and sample instability meant no useful data was obtained.

C Rare successful scan of a vibrodissociated neuron. Patch recording was attempted from this cell, but no seal was obtained.

Vibrodissociation has the virtue of bringing live cells within reach of the SICM pipette. Moreover, it carries an additional benefit in the case of the cerebellar interneuron-Purkinje system that we are interested in, at least in rats:⁴ Purkinje cells possess functional NMDARs only transiently, in early development (Farrant and Cull-Candy 1991; Llano et al. 1991b; Rosenmund et al. 1992). NMDA-sensitive phenomena recorded from a vibrodissociated rat Purkinje cell must therefore be mediated presynaptically. Since the rest of the presynaptic cell is absent the NMDARs must be located on or very near the synaptic bouton (Duguid et al. 2007).

However, vibrodissociation produces a lot of floating debris that can easily block or transiently interact with the hopping pipette to create artefacts. The dissociated cells tend not to be well stuck down, and often retain stubs of dendrite that flap around as the dish moves, confounding the scan. The majority of scans attempted from vibrodissociated cells look akin to the one in figure 2B. There have been a small minority of more successful attempts, such as that in figure 2C. If a way can be found to increase the hit rate, this preparation is rather promising.

For the time being, however, the main effort will concentrate on dissociated cultures. These are relatively stable and well-behaved; after many months practice, they can now be scanned with relative confidence.

3.3 Localised patching from neural structures using ‘hopping mode’ SICM

With the provisos mentioned above, the hopping probe gives us the ability to image complex networks of neurons. It should therefore be possible to record from known positions in such networks using the smart patch technique. An example is shown in figure 3A-B. Here, the cells concerned were cultured rat hippocampal neurons, and a recording was made from an apical dendrite.

Seal formation using the SICM pipette is largely the same as in traditional patch clamping (Sakmann and Neher 1995): the pipette is slowly lowered to the surface while monitoring resistance, and suction applied once the membrane is reached. One notable difference is that the pipette and approach are perfectly vertical, rather than coming in from the side as is common when using a normal micromanipulator. For this reason, as well as the small size of the pipette tip, it is not possible to guide the pipette by eye, so we rely on the seal test pulse in combination with the previously-measured surface height to gauge proximity.

In practice, we find that obtaining a gigaseal is actually easier with SICM than by traditional methods. This is probably in large part due to the vertical approach, along with the relative stability of the piezo actuator and the tiny, precise movements that it makes possible.

SICM pipettes are significantly smaller than those used for most conventional patching, even for single channel methods. Given this, there was some concern that we would be sampling too small an area to consistently pick up channel activity. This worry appears to be unfounded: even with very small patches, there are usually some channels to be found, sometimes too many.

Because of the small size, it is not practical to polish the pipette tip, as is usual for patch clamping, but this does not seem to hinder seal formation. The originators of the smart patch technique also chose not to use any Sylgard coating (Gorelik et al. 2002). However, here, it does contribute to a reduction in pipette noise, but due to their very small size, the pipette tips are prone to block when the Sylgard is cured. As a consequence, significantly more pipettes are discarded this way—as many as 80%, compared to 30% when they are uncoated.

⁴In mice, by contrast, Purkinje cells retain functional NMDARs into adulthood (Renzi et al. 2007).

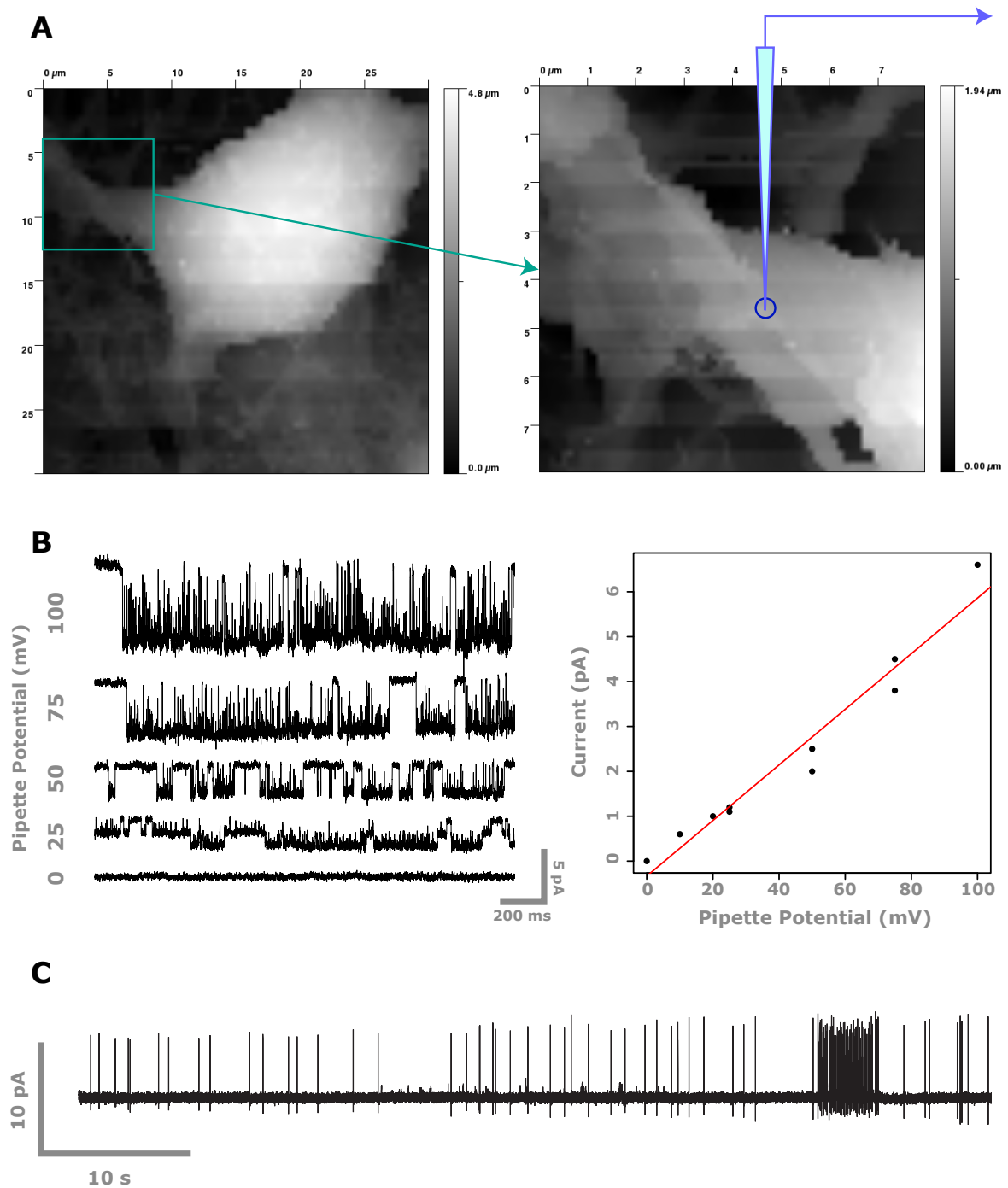


Figure 3 Cell attached recordings from neuronal processes using the smart patch technique. **A** Cultured rat hippocampal cells were scanned in hopping mode and a dendritic location chosen for recording. The scanning pipette was then lowered to the cell surface and suction applied to form a seal. **B** Single channel openings (left) and an I-V fit derived from this recording (right); estimated channel conductance was 62 pS. **C** Cell attached spike train recording from a similar location on another cell (scans not shown).

The high resistance of the pipettes also generally restricts recording to the cell-attached mode: the tip is too small to permit the patch to be broken for whole-cell recording, and the high access resistance would lead to problems with noise, clamping and time resolution. Therefore, this study focusses on cell-attached single channel recording. As seen in figure 3C, we have also been able to record action potential firing in cell-attached mode.

It is important to note that the recordings shown in figure 3 were from arbitrary locations. While SICM can image fine structures in the network, the topography alone is insufficient to unambiguously identify them.

To draw conclusions from positional recordings, it is important to be able to demonstrate that they are taken from relevant structures. We must repeatedly be able to locate the right regions on cells, and record from them enough times to build a case. To do so requires additional tools.

3.4 Combining SICM with optical techniques

The SICM apparatus rests on the stage of an inverted microscope, allowing the sample to be visualised from below at the same time as being scanned from above. In this position, it obstructs the normal brightfield illumination pathway. The space occupied leaves no room for the phase rings that would normally increase contrast in transparent samples such as cell cultures. As a result, although brightfield imaging is possible, the visual quality tends to be very poor.

To identify our targets more clearly, we must turn instead to fluorescent markers. We have so far focussed mainly on two such markers, with the aim of using them in combination.

Styryl dyes such as FM 1-43 and SynaptoRed (FM 4-64) are amphipathic molecules that readily enter and leave the plasma membrane but are prevented by their permanent charge from passively diffusing across it. They are barely fluorescent in water, but fluoresce strongly when partitioned into the hydrophobic environment of the membrane. They can thus be used as markers of vesicular activity (Betz et al. 1992; Ryan 2001; Brumback et al. 2004). The membrane is first stained with the dye and then, after a short delay, washed out. Patches of membrane that have been endocytosed in the interim retain their fluorescence, and these internalised puncta can be taken to identify presynaptic release sites. Endocytosis can be stimulated during loading by depolarising the cells, although we typically find a reasonable level of marking even with only spontaneous activity.

GAD65-GFP transgenic mice express green fluorescent protein (GFP) under the control of the promoter for the 65 kDa isoform of glutamic acid decarboxylase (GAD65), an enzyme that catalyses the production of γ -aminobutyric acid (GABA) (López-Bendito et al. 2004). This leads to selective GFP expression in inhibitory neurons, and specifically the cerebellar interneurons we are interested in. The resulting fluorescence is bright, stable and distinct.

FM 1-43 does not combine well with GFP because the two emission spectra overlap considerably, but SynaptoRed and GFP are well separated, showing virtually no crosstalk. They are thus well-suited to use together to help target recordings.

An example is shown in figure 4. In this case, the red puncta coincide with the intersection between two interneuron processes, a fine meandering one taken to be an axon, and a thicker dendrite coming directly from the cell visible in the main picture of A. The scan in B shows quite a number of processes passing through this spot, but we can pick out the axon by inspection and target the pipette to it.

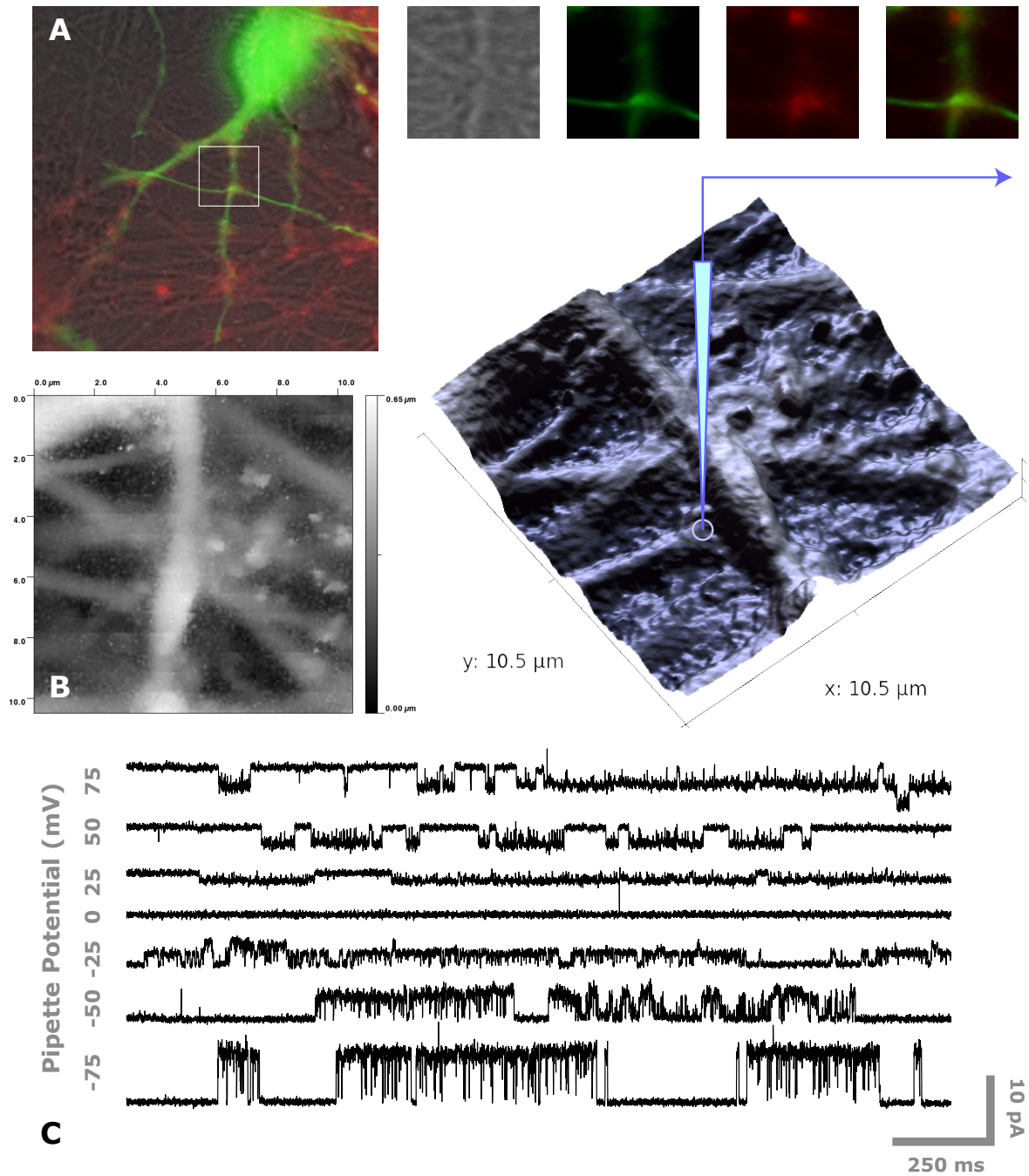


Figure 4 Combining SICM with fluorescence to target patch recording.

A Composite optical image of transgenic mouse cerebellar culture, showing brightfield (grey), expressed GFP (green) and SynaptoRed labelling (red). The square outlined in white is expanded at the right to show the separate channels and a composite of the fluorescent markers only.

B Hopping mode image of the same region, shown in flat and 3D renderings. By comparing with the optical images, a potential presynaptic region can be selected for patch recording.

C Single channel recordings from such a putative presynaptic patch (not the same cell).

Note that, although the optical micrographs are taken with a 100 $\times$ oil immersion objective, the most powerful we have, the resolution is still not high enough to make identification of the synapse trivial. Nevertheless, with the aid of these fluorescent markers, it should now be possible to make recordings that we can be confident come from interneuron terminals.

4 Discussion & future work

This preliminary study shows that the hopping mode protocol of SICM allows the imaging of complex neuronal networks and recording from specific positions within them. Identifying the locations to be targeted is not trivial, but can be achieved by combining multiple fluorescent markers with the topographic data obtained from SICM. These processes are by no means perfect: there are numerous points of potential failure and many refinements still to be made. In essence, though, the approach works. The goal now is to *use* it.

The recordings made to date have been concerned with establishing the feasibility of the techniques rather than gathering data pertaining to a biological question. To move to the latter, we must incorporate some tools of classical pharmacology.

NMDARs are well characterised electrophysiologically and pharmacologically, and a number of agents are available to help distinguish them (Watkins and Olverman 1987). The defining agonist is, of course, NMDA. There are selective antagonists such as AP5, and the channel's characteristic Mg^{2+} block. In addition to the voltage-dependence of this block, there is evidence that at least some subunit combinations exhibit an intrinsic voltage-dependence of their own (Clarke and Johnson 2008).

While it is unrealistic to expect to silence all other channels that may be present in an axon terminal, fairly broad-spectrum blockers such as tetraethylammonium (TEA) and 4-aminopyridine (4AP) for potassium channels, along with tetrodotoxin (TTX) for voltage-gated sodium channels, should reduce extraneous currents and help focus on any NMDAR signal.

If NMDARs are present, the ability to detect them will depend on their number and distribution around the terminal. For example, if they are restricted to the synaptic cleft they will never be accessible to the pipette. But there is currently no evidence of such localisation, so it makes sense to start from the 'uninformative' assumption that the channels could be anywhere on the terminal with equal likelihood.

Estimates of the sizes of inhibitory terminals in the cerebellum vary. If we use the upper end of the range observed by Lemkey-Johnston and Larramendi (1968), 2 μm diameter, and model the bouton as a hemisphere, then a patch taken with a pipette tip of radius 100 nm will sample $\sim 0.3\%$ of the surface area.⁵ At this rate of sampling we would need ~ 1000 recordings to make it more than 95% likely that we have sampled everything in the membrane. However, this value drops rapidly if there are multiple targets. If there were, on average, 50 NMDARs in terminals of this size, then we would be 95% certain to find one with 20 recordings. It is thus plausible that we will be able gather sufficient data to draw some conclusions about the NMDAR population in terminals.

Even if we do find NMDARs, the 'unnatural' nature of synapses in culture may leave the finding open to question. Ultimately, being able to generalise the technique to other preparations

⁵This is certainly a conservative estimate. Lemkey-Johnston and Larramendi's figures are for the major axes of elliptical cross sections, so the actual areas would be smaller. Conversely, the patch area is here calculated as flat, whereas in reality there is a deformation of the membrane into the pipette leading to a larger patch area (Sakmann and Neher 1995).

would greatly improve its utility and persuasiveness. The difficulties with acute slices are such that we do not foresee being readily able to do SICM-based recording from them in the near future. The nerve-bouton preparation, however, can probably be made useable, and that would be the obvious next port of call if and when we exhaust the possibilities of cultured cells.

Among improvements that still need to be made to the system as it stands, the most urgent is probably the ability to apply perfusion without unduly disrupting scanning. Better cell health is the main goal, but the capacity to perform bath application of drugs might also open up some useful experimental options.

Although SICM is normally restricted to cell-attached recording by the small pipette tip size required for high resolution scanning, there may be ways around this. One suggestion that is yet to be put into practice is to enlarge the pipette tip by deliberately breaking the end once the scan has been made, and then patch with this enlarged tip. It is not clear that this can be done with any consistency, but some anecdotal reports from our colleagues at Imperial suggest that it may. If so, this would considerably increase the scope of experiments that could be performed.

The piezo-based scanning used in SICM is well-suited to simultaneous confocal imaging, and we are some way towards building a two laser imaging system on the rig to support this. Although there are a number of significant problems remaining to be ironed out, notably that of correctly aligning the confocal spot with the SICM pipette tip, once complete this should greatly improve our ability to correlate fluorescent and topographic images, allowing the target boutons to be located more easily and with greater confidence.

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References

- Norio Akaike and Andrew J Moorhouse. Techniques: applications of the nerve-bouton preparation in neuropharmacology. *Trends Pharmacol Sci*, 24(1):44–7, Jan 2003.
- Phillippe Ascher and Linda Nowak. The role of divalent cations in the N-methyl-D-aspartate responses of mouse central neurones in culture. *J Physiol*, 399:247–66, May 1988.
- William J Betz, Fei Mao, and Guy S Bewick. Activity-dependent fluorescent staining and destaining of living vertebrate motor nerve terminals. *J Neurosci*, 12(2):363–75, Feb 1992.
- Stephan D Brenowitz and Wade G Regehr. Calcium dependence of retrograde inhibition by endocannabinoids at synapses onto Purkinje cells. *J Neurosci*, 23(15):6373–84, Jul 2003.
- Audrey C Brumback, Janet L Lieber, Joseph K Angleson, and William J Betz. Using FM1-43 to study neuropeptide granule dynamics and exocytosis. *Methods*, 33(4):287–294, Aug 2004. doi: 10.1016/j.ymeth.2004.01.002.
- Jason M Christie and Craig E Jahr. Dendritic NMDA Receptors Activate Axonal Calcium Channels. *Neuron*, 60(2):298–307, Oct 2008. doi: 10.1016/j.neuron.2008.08.028.
- Richard J Clarke and Jon W Johnson. Voltage-dependent gating of NR1/2B NMDA receptors. *J Physiol*, 586(23):5727–5741, Oct 2008. doi: 10.1113/jphysiol.2008.160622.
- Marco A Diana, Carole Levenes, Ken Mackie, and Alain Marty. Short-term retrograde inhibition of GABAergic synaptic currents in rat Purkinje cells is mediated by endogenous cannabinoids. *J Neurosci*, 22(1):200–8, Jan 2002.
- Raymond Dingledine, editor. *Brain Slices*. Plenum Press, New York, 1984.
- Ian C Duguid and Trevor G Smart. Presynaptic NMDA receptors. In Antonius M VanDongen, editor, *Biology of the NMDA receptor*, chapter 14, pages 313–328. CRC Press, Boca Raton, 2008.
- Ian C Duguid and Trevor G Smart. Retrograde activation of presynaptic NMDA receptors enhances GABA release at cerebellar interneuron–Purkinje cell synapses. *Nat Neurosci*, 7(5): 525–533, May 2004. doi: 10.1038/nn1227.
- Ian C Duguid, Yuriy Pankratov, Guy W J Moss, and Trevor G Smart. Somatodendritic Release of Glutamate Regulates Synaptic Inhibition in Cerebellar Purkinje Cells via Autocrine mGluR1 Activation. *J Neurosci*, 27(46):12464–12474, Nov 2007. doi: 10.1523/JNEUROSCI.0178-07.2007.
- Mark Farrant and Stuart G Cull-Candy. Excitatory amino acid receptor-channels in Purkinje cells in thin cerebellar slices. *Proc Biol Sci*, 244(1311):179–84, Jun 1991. doi: 10.1098/rspb.1991.0067.
- Maike Glitsch. Calcium influx through N-methyl-d-aspartate receptors triggers GABA release at interneuron–Purkinje cell synapse in rat cerebellum. *Neuroscience*, 151(2):403–409, Jan 2008. doi: 10.1016/j.neuroscience.2007.10.024.
- Julia Gorelik, Yuchun Gu, Hilmar A Spohr, Andrew I Shevchuk, Max J Lab, Sian E Harding, Christopher R W Edwards, Michael Whitaker, Guy W J Moss, David C H Benton, Daniel Sanchez, Alberto Darszon, Igor Vodyanoy, David Klenerman, and Yuri E Korchev. Ion channels in small cells and subcellular structures can be studied with a smart patch-clamp system. *Biophys J*, 83(6):3296–303, Dec 2002.

- Yuchun Gu, Julia Gorelik, Hilmar A Spohr, Andrew I Shevchuk, Max J Lab, Sian E Harding, Igor Vodyanoy, David Klenerman, and Yuri E Korchev. High-resolution scanning patch-clamp: new insights into cell function. *FASEB J*, 16(7):748–50, May 2002. doi: 10.1096/fj.01-1024fje.
- P K Hansma, B Drake, O Marti, S A Gould, and C B Prater. The scanning ion-conductance microscope. *Science*, 243(4891):641–3, Feb 1989.
- Bertil Hille. *Ion channels of excitable membranes*, 3rd edition. Sinauer Associates, Sunderland Massachusetts, 2001.
- Masanobu Kano, U Rexhausen, J Dreessen, and Arthur Konnerth. Synaptic excitation produces a long-lasting rebound potentiation of inhibitory synaptic signals in cerebellar Purkinje cells. *Nature*, 356(6370):601–4, Apr 1992. doi: 10.1038/356601a0.
- Gerald Allan Kerkut and Howard V Wheal, editors. *Electrophysiology of isolated mammalian CNS preparations*. Academic Press, London, 1981.
- Yuri E Korchev, C Lindsay Bashford, Mihailo Milovanovic, Igor Vodyanoy, and Max J Lab. Scanning ion conductance microscopy of living cells. *Biophys J*, 73(2):653–8, Aug 1997a.
- Yuri E Korchev, Mihailo Milovanovic, C Lindsay Bashford, D C Bennett, Elena V Sviderskaya, Igor Vodyanoy, and Max J Lab. Specialized scanning ion-conductance microscope for imaging of living cells. *J Microscopy*, 188(Pt 1):17–23, Oct 1997b.
- Anatol C Kreitzer, Adam G Carter, and Wade G Regehr. Inhibition of interneuron firing extends the spread of endocannabinoid signaling in the cerebellum. *Neuron*, 34(5):787–96, May 2002.
- N Lemkey-Johnston and L M H Larramendi. Types and distribution of synapses upon basket and stellate cells of the mouse cerebellum: an electron microscopic study. *J Comp Neurol*, 134(1):73–111, Jan 1968. doi: 10.1002/cne.901340106.
- Carole Levenes, Hervé Daniel, and Francis Crepel. Retrograde modulation of transmitter release by postsynaptic subtype 1 metabotropic glutamate receptors in the rat cerebellum. *J Physiol*, 537(Pt 1):125–40, Nov 2001.
- Isabel Llano, Nathalie Leresche, and Alain Marty. Calcium entry increases the sensitivity of cerebellar Purkinje cells to applied GABA and decreases inhibitory synaptic currents. *Neuron*, 6(4):565–74, Apr 1991a.
- Isabel Llano, Alain Marty, Clay M Armstrong, and Arthur Konnerth. Synaptic- and agonist-induced excitatory currents of Purkinje cells in rat cerebellar slices. *J Physiol*, 434:183–213, Mar 1991b.
- Guillermina López-Bendito, Katherine Sturgess, Ferenc Erdélyi, Gábor Szabó, Zoltán Molnár, and Ole Paulsen. Preferential Origin and Layer Destination of GAD65-GFP Cortical Interneurons. *Cerebral Cortex*, 14(10):1122–1133, Apr 2004. doi: 10.1093/cercor/bhh072.
- Alain Marty and Isabel Llano. Modulation of inhibitory synapses in the mammalian brain. *Curr Opin Neurobiol*, 5(3):335–41, Jun 1995.
- Pavel Novak, Chao Li, Andrew I Shevchuk, Ruben Stepanyan, Matthew Caldwell, Simon Hughes, Trevor G Smart, Julia Gorelik, Max J Lab, Guy W J Moss, Gregory I Frolenkov, David Klenerman, and Yuri E Korchev. Nanoscale live cell imaging using hopping probe ion conductance microscopy. *Nat Met*, (in press), 2009.
- Thomas A Pitler and Bradley E Alger. Postsynaptic spike firing reduces synaptic GABAA responses in hippocampal pyramidal cells. *J Neurosci*, 12(10):4122–32, Oct 1992.

- Massimiliano Renzi, Mark Farrant, and Stuart G Cull-Candy. Climbing-fibre activation of NMDA receptors in Purkinje cells of adult mice. *The Journal of Physiology*, 585(1):91–101, Oct 2007. doi: 10.1113/jphysiol.2007.141531.
- Christian Rosenmund, Pascal Legendre, and Gary L Westbrook. Expression of NMDA channels on cerebellar Purkinje cells acutely dissociated from newborn rats. *J Neurophys*, 68(5):1901–5, Nov 1992.
- Timothy A Ryan. Presynaptic imaging techniques. *Curr Opin Neurobiol*, 11(5):544–9, Oct 2001.
- Bert Sakmann and Erwin Neher, editors. *Single Channel Recording, 2nd edition*. Plenum Press, New York, 1995.
- Jung Hoon Shin, Yu Shin Kim, and David J Linden. Dendritic glutamate release produces autocrine activation of mGluR1 in cerebellar Purkinje cells. *PNAS*, 105(2):746–50, Jan 2008. doi: 10.1073/pnas.0709407105.
- Bela Szabo, Michal J Urbanski, Tiziana Bisogno, Vincenzo Di Marzo, Aitziber Mendiguren, Wolfram U Baer, and Ilka Freiman. Depolarization-induced retrograde synaptic inhibition in the mouse cerebellar cortex is mediated by 2-arachidonoylglycerol. *The Journal of Physiology*, 577(1):263–280, Sep 2006. doi: 10.1113/jphysiol.2006.119362.
- P Vincent, Clay M Armstrong, and Alain Marty. Inhibitory synaptic currents in rat cerebellar Purkinje cells: modulation by postsynaptic depolarization. *J Physiol*, 456:453–71, Oct 1992.
- V S Vorobjev. Vibrodissociation of sliced mammalian nervous tissue. *J Neurosci Met*, 38(2-3): 145–50, Jul 1991.
- Jeffrey C Watkins and Henry J Olverman. Agonists and antagonists for excitatory amino acid receptors. *Trends Neurosci*, 10(7):265 – 272, 1987. doi: DOI:10.1016/0166-2236(87)90171-8.
- Takayuki Yoshida, Kouichi Hashimoto, Andreas Zimmer, Takashi Maejima, Kenji Araishi, and Masanobu Kano. The cannabinoid CB1 receptor mediates retrograde signals for depolarization-induced suppression of inhibition in cerebellar Purkinje cells. *J Neurosci*, 22(5):1690–7, Mar 2002.