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Measuring action potential-evoked transmission at individual synaptic contacts

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Abstract

In the neuronal culture experimental system, the total synaptic connection between two neurons can consist of large numbers of synaptic sites, each behaving probabilistically. Studies of synaptic function with paired recordings typically consider the summed response across all of these sites and from this infer the average response. Understanding of synaptic transmission and plasticity could be improved by examination of activity at as few synaptic sites as possible. To this end, we develop a system for recording responses from individual contacts. It relies on a precisely regulated pneumatic/hydrostatic pressure system to create a microenvironment within which individual synapses are active, and an acoustic signature method to monitor the stability of this microenvironment noninvasively. With this method we are able to record action potential-evoked postsynaptic currents consistent with individual quanta. The approach does not distort synaptic current waveforms and permits stable recording for several hours. The method is applied to address mechanisms of short-term plasticity, the variability of latency at individual synaptic sites and, in a preliminary experiment, the independence of nearby synapses on the same axon.

Introduction

Cell culture is an important research tool in neurobiology because the preparation is well suited to measurement using imaging (Ehlers *et al* 2007), electrophysiology (Lau and Bi 2005) or molecular assays (Linhoff *et al* 2009). Cultured neurons and glia are also subject to precise manipulation using subcellular genetic tools (Barrett *et al* 2006), microdissection (Chang *et al* 2006), patterned control of cell growth (Houchin-Ray *et al* 2009), microfluidics (Taylor *et al* 2010) and other methods. In any of these experimental approaches, investigators may wish to monitor synaptic function. However, although the overall synaptic

density in culture is generally lower than in the brain (Schikorski and Stevens 1997), neurons in culture have fewer potential synaptic partners, resulting in the formation of increased numbers of synaptic contacts between pairs of neighboring cells. As a result, electrophysiological recordings from the total direct synaptic connection of two neurons in culture can reflect transmission at any combination of dozens of synaptic boutons located anywhere from the soma to the distal dendrites of the postsynaptic cell. Differences in functional properties of individual contacts, likely to be large (Rosenmund *et al* 1993), cannot usually be resolved in somatic recordings. Dendritic distance influences such factors as the number of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors at postsynaptic densities (Smith *et al* 2003) and response to a spike-timing-dependent plasticity protocol (Froemke *et al* 2005), as well as passive cable properties (Prinz and Fromherz 2003) and active conductances (George *et al* 2009), so summation of responses across sites will permit determination only of net values. Selectively permitting activity at small numbers of synaptic sites has the potential to allow more accurate inference of functional parameters. Such a restriction to a focal region of the preparation was performed at the neuromuscular junction to determine definitively that calcium was not necessary for the propagation of the nerve impulse but was required for neurotransmitter release (Katz and Miledi 1965). It has also been used to measure the degree of saturation of glutamate receptors (McAllister and Stevens 2000) and to determine the spatial specificity of dendritic protein synthesis (Sutton *et al* 2006).

To record action potential (AP)-evoked transmission with quantal resolution, improved stability, non-distortion of responses and capacity for noninvasive monitoring, we developed the method presented here. Conceptually simple, the technique is suitable for any study requiring modification of the subcellular environment. Experiments made possible with the method indicate that depletion of a synaptic resource such as readily releasable neurotransmitter vesicles is not a primary determinant of short-term plasticity, that individual synaptic currents transmit little record of recent activity beyond the previous AP, and that adjacent synapses on the same axon may function independently as theoretical studies have predicted.

A protocol is provided in the supplementary material available at stacks.iop.org/JNE/9/036014/mmedia.

Methods

Culture technique

Hippocampal neurons from embryonic rat (E18–E20) were cultured as previously reported (Wilcox *et al* (1994) with modifications⁶). The changes were designed to achieve optimal neuronal density for microperfusion experiments and dendritic splay sufficient for finding isolated axonal-dendritic connections on beds of glial cells. Poly-L-lysine (1 mg ml⁻¹ in H₂O, Peninsula Labs) was stamped using custom stamps (Allegheny Marking) or sprayed using an Airbrush (Aztec) onto 12 mm #0 cover slips (Karl Hecht; supplementary figure S1 available at stacks.iop.org/JNE/9/036014/mmedia). Cover slips were dried for 24 h, soaked

⁶https://jshare.johnshopkins.edu/dnauen1/public_html/

for 24 h in culture water and dried again before pre-incubation with the culture medium for 12 h prior to cell plating. Cells were plated at $15\,000\text{--}25\,000\text{ ml}^{-1}$, with plating done only once per culture batch, and batches generated once per week. Given the size of the polylysine ‘islands’ ($\sim 0.1\text{--}0.9\text{ mm}^2$), this density results in 5–15 neurons/island. At the age at which cells were used for experiments, 11–17 DIV, this results in a distance of $\sim 3\text{ }\mu\text{m}$ along a given dendrite between boutons formed by the same presynaptic neuron, as confirmed by preliminary experiments with FM 1–43 synaptic labeling (see McAllister and Stevens (2000) for inter-bouton distances in comparable culture conditions). Mitotic inhibitors such as cytosine arabinoside were not used. All experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Patch-clamp technique

Perforated whole-cell patch-clamp (Rae *et al* 1991) is well suited to long-duration plasticity experiments, and is also ideal for maintaining gigaohm seals in the low calcium external solution used in microperfusion experiments to prevent evoked release. $3\text{ }\mu\text{l}$ of amphotericin B (Sigma, $40\text{ }\mu\text{g }\mu\text{l}^{-1}$ in dimethyl sulfoxide) was mixed with $600\text{ }\mu\text{l}$ internal solution and dissolved thoroughly, and then back filled into pipettes (Kimax, pulled to $2.0\text{--}2.2\text{ M}\Omega$) after tip-fill with amphotericin-free internal solution. The internal solution contained (in mM, Fisher Scientific) K-gluconate 136.5, KCl 17.5, NaCl 9, $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$ 1, HEPES 10 and EGTA 0.2. The external bath solution contained NaCl 150, KCl 3, $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ 3, $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$ 2, HEPES 10 and glucose 5. Seals were obtained in the normocalcemic solution on cells with processes oriented roughly perpendicular to the direction of global perfusion flow. For microperfusion (below), the global bath solution was as above but $[\text{Mg}^{2+}]$ was increased to 4.8 mM and $[\text{Ca}^{2+}]$ was decreased to 0.2 mM, whereas the local perfusion solution was identical in composition to the original global bath solution. Internal and external solutions were titrated to an osmolarity of 328–9 mOsm at pH 7.4. All experiments were performed at room temperature ($21\text{--}23\text{ }^\circ\text{C}$).

Electrophysiology and analysis

Stimulation and recording was performed with an Axon 700B amplifier (Molecular Devices). Sampling was at 10 kHz and filtering was with a 4-pole Bessel at 3 kHz. Trains of three or four APs were evoked in the presynaptic cell by stepping the holding voltage to the suprathreshold potential for 1–1.5 ms, with both neurons otherwise voltage-clamped at -70 mV . Successful initiation of each AP was verified by observation of the action current. In each trial, the penultimate inter-pulse interval (IPI) was varied among the possible values 25, 50, 100, 200 or 400 ms, with the assignment random but constrained such that no AP train timing was presented $n + 1$ times until all timings were presented n times. 50 ms was fixed as the duration of the other IPI (3-pulse trains) or IPIs (4-pulse trains). The amplitude of the postsynaptic current (PSC) was measured by finding the single lowest value for the membrane current in a window after the presynaptic AP and taking the mean over samples from 0.3 ms before to 1 ms after this trough. Noise fluctuations generally led to values of a few pA in cases when no PSC occurred. Success or failure of synaptic transmission was determined using a template-match method (Clements and Bekkers 1997). For some analyses, synaptic current waveforms were fit by a template having three terms, amplitude, rise time constant and decay time constant, $A(1 - e^{-t/\tau_{\text{rise}}})e^{-t/\tau_{\text{decay}}}$ (see Bekkers and Stevens

1996 and Nielsen *et al* 2004 for related equations for fitting synaptic currents). Binomial confidence intervals were obtained using the Agresti–Coull method (Agresti and Coull 1998). Fisher’s exact test was used to assess significance of binomial comparisons. Kolmogorov–Smirnov tests were used to compare cumulative distributions. In some figures, membrane current traces are smoothed for display using a 1 ms moving average. For clarity, the negative symbol is omitted from labels of the ordinate of most membrane current plots. All experimental and analysis software, which is written in Matlab (Mathworks), is available for download⁷.

Single-cell labeling and imaging

Experiments could be done blindly or with synaptic labeling (supplementary figure S2 available at stacks.iop.org/JNE/9/036014/mmedia). For labeling, SynptoGreen (Biotium) was perfused at 12.5 μM in the normocalcemic solution supplemented with 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Sigma) to block AMPAR current. The presynaptic cell was stimulated to fire APs at a mean rate of 5 Hz. Seven trains of 25 pulses were evoked with 1 min between trains and following the final train. Loading was followed by rinse with a low-calcium bath solution and Advasep-7 (Biotium). Because only one cell was stimulated, only boutons from that cell were labeled, simplifying the search for synaptic contacts between the patched cells.

When performed, single-neuron labeling (Murthy *et al* 1997) facilitated the task of finding isolated boutons (Liu and Tsien 1995). Moderate intensity boutons were selected (Chen *et al* 2004) as these were considered more likely to contain only a single active zone. The drawback to single-neuron labeling was that it required maintenance of high-quality patches during fairly intense stimulation in the presence of dye, which could compromise seal quality slightly, and it delayed the start of recording. As an alternative, when a single, properly oriented dendrite was visible, it was straightforward to search for synaptic contacts in low Ca^{2+} bulk perfusion solution without labeling by guiding the positive perfusion tip (below) along the length of the dendrite while stimulating the presynaptic neuron. When a PSC was seen, the negative tip was used to isolate responses. In this way visual information about the bouton, such as size, was unavailable, but this was often offset by higher patch quality and longer recording durations. See ‘Labeling’ in supplementary material for additional information.

Microperfusion

For focal perfusion, positive pneumatic pressure (2 or 3 psi each experiment) was delivered to a fine-tipped micropipette containing normocalcemic extracellular solution. This solution passed over a small region of tissue before being captured in a pipette transmitting negative pressure created by a hybrid siphon/vacuum system. The siphon flowed to a collection point beside the stage before being removed by a suction line (figures 1(A) and (B)). With this configuration, much lower and more finely regulable negative pressures were obtained than would be possible with suction alone. This yielded a markedly improved resolution, with recorded currents comparable to individual quantal events. Preliminary experiments with

⁷https://jshare.johnshopkins.edu/dnauen1/public_html/

dyes (Trypan Blue, Sigma, and SynaptoGreen, Biotium, which fluoresces only if it contacts a lipid membrane) were used to fine-tune angles, check perfusion of the glial bed and confirm that the negative tip traps all of the locally applied solution. Headstages and recording electrodes were mounted on manipulators in the upper-left/lower-right corners of the stage, while the microperfusion tip holders were placed in the lower left and upper right, with axes of movement allowing independent modification (supplementary figure S3).

Using water as the bulk perfusion solution during initial configuration of the system, the extracellular bath solution used in the microperfusion stream became visible with phase-contrast optics (figure 1(C)). To assess the physical stability of the system, we performed time-lapse imaging. Pipette tip positions and stream characteristics were highly stable for at least 80 min (supplementary movies 1–3 available at stacks.iop.org/JNE/9/036014/mmedia). During time-lapse imaging of patch-clamp experiments, a small amount of thermal drift of the patch pipettes on the cell bodies is generally observed. We compared the stability of the patch pipettes (supplementary movie 4), which was sufficient for recordings of 2 h or more, to that of the microperfusion pipettes and found that the latter appeared equally or more stable over time. Microperfusion permitted restriction of evoked exocytosis to a small subset of release sites active during standard perfusion (figure 1(D)). For an initial assessment of the extent to which the size of PSCs in microperfusion was reduced due to dendritic cable properties (Prinz and Fromherz 2003), we applied microperfusion directly at the soma in a test experiment, finding that microperfusion events were small independent of dendritic filtering (supplementary figure S4(A)). See ‘Configuring the local perfusion system’ in supplementary material for additional information.

Measuring flow with acoustic signature

The microperfusion stream was not visible on phase contrast optics under experimental conditions, so optical monitoring was not possible in the absence of potentially toxic or neuroactive dye. To confirm noninvasively that siphon outflow, and hence negative microperfusion pressure, was constant during each experiment, the sound created as the perfusate was removed from the siphon outflow by the vacuum line was recorded simultaneously with electrophysiological data (figure 2) with a precision microphone (PCB Piezotronic) placed near the aperture in the housing for the siphon–suction junction (figures 2(A) and (B)). The complex waveforms of the sound made automated methods such as harmonic regression unsatisfactory in detecting changes (not shown), but visual inspection of the audio signal was a reliable indicator of block, permitting detection of interruption of flow due to an experimenter-induced obstruction (figure 2(D)). When siphon outflow height is varied to increase or decrease negative pressure, changes in the acoustic signature are seen, confirming that the method permits detection of small changes in siphon flow (figure 2(E)). In the setting of stable global perfusate flow and depth and stable tip positions, stable microperfusion flow indicates microenvironment stability. See ‘Siphon control’ in supplementary material available at stacks.iop.org/JNE/9/036014/mmedia for additional information.

Results

To confirm that microperfusion of the normocalcemic solution was necessary for release of transmitter and thus that release originated from within the microperfusion stream, we removed the positive pressure to the microperfusion system for individual trials (figure 3(A)). Without local perfusion no responses were seen, and when local perfusion was resumed, responses returned as soon as could be assessed. At this synapse, as at most synapses studied, paired-pulse ratio (ratio of second response to first response) tended to be greater after brief IPIs (figure 3(A), inset; averages of 14 trials each). Thus, had positive pressure not been stopped the second trial would have been at least as likely as the first and third trials to result in PSCs. Turning off microperfusion reliably silenced release at all synapses tested. The rapid appearance and disappearance of synaptic events when calcium micropuffing was turned on and off, respectively, indicate that the puffed calcium is necessary for transmitter release and acts directly at the synapse.

To confirm the functional stability of the configuration, we recorded postsynaptic responses to brief trains of three to four presynaptic APs at inter-trial intervals of 45 s for periods of 20–60 min. As expected due to the stochastic character of synaptic function, PSC responses varied from trial to trial (figure 3(B)), but no trend was observed. To quantify stability we fit the values linearly. The resulting slopes range from -0.0868 to 0.0867 , with a mean of 0.0015 . The mean of the distribution of slopes is not likely to be different from 0 ($p > 0.9$, t -test). The t -test comparison of the first half versus the second half of each recording yielded no significant difference ($p > 0.4$). To test the contribution of the negative pressure component of the microperfusion system to the observed fluctuations in responses, that component was not used in one experiment (figure 3(B), last panel). Responses to presynaptic APs at this synapse showed similar fluctuation to others, confirming that the fluctuations are not due to variations in negative pressure but rather to variability at the synapse itself. Because the negative pressure component of the microperfusion system was generally necessary for responses consistent with single or few synaptic sites, negative pressure was in place during all reported experiments apart from this test. See supplementary Material available at stacks.iop.org/JNE/9/036014/mmedia for further discussion of the need for negative pressure.

Glutamate receptor antagonists have been used in some localized activation studies to block synaptic currents at non-enabled sites, but if intermixture of the global solution into the locally perfused area occurs, this could alter the waveforms recorded from the active site. Conversely, the reduction of calcium for disabling synaptic release, as done here, would not be expected to affect waveforms recorded in the voltage-clamped postsynaptic neuron, even in the event of intermixture. To test for distortion in our system, we compared responses from four classes of experiments: standard perfusion at a very weak synaptic connection (AP-evoked), localized perfusion (AP-evoked), local application and removal of hyperosmotic solution using the microperfusion system (hyperosmolarity-evoked), and during bath application of the sodium channel antagonist tetrodotoxin (spontaneous; figure 3(C)). Events recorded under the four conditions were similar (figure 3(D)), indicating that microperfusion does not distort waveforms. The distribution of event sizes recorded under

conditions of AP block by TTX, often taken as an indicator of the quantal size, is comparable to that of the microperfusion PSCs.

To determine the latency of exocytosis at locally perfused synapses, the interval between the presynaptic AP and the onset of the PSC was measured. The AP onset was taken to be the first down-going sample in the action current notch of the presynaptic voltage clamp trace and the PSC onset was taken as the time of the peak in the template-fit detection criterion (Clements and Bekkers 1997; figure 4(A)) if the detection threshold was exceeded. Apart from occasional biphasic cases (discussed below), values for latency were narrowly distributed (figure 4(B)), though they showed long tails consistent with the possibility of asynchronous release. There was no clear trend for latencies to become longer or shorter over the successive responses within each stimulus train (figure 4(B)). In infrequent cases (2 of 105 pairs of neurons), PSCs had two components separated by large and consistent differences in latency (figure 4(B)). These biphasic responses may reflect the presence of two separate axonal branches of the presynaptic neuron within the focal perfusion stream. The sites presumably lie at different axonal distances from the presynaptic soma and thus exhibit different latencies. The widths of the latency distributions were comparable around each peak to the monophasic distributions observed at other synapses.

In the short term, both facilitating and depressing processes are likely to occur at central synapses (Zucker and Regehr 2002). Mechanisms of short-term facilitation such as calcium accumulation in the presynaptic terminal are release-independent. On the other hand, the most commonly considered cause for depression is vesicle depletion, which requires release. With few release sites, if depletion affects transmission, the depletion due to a successful release would render release after a second AP less likely. The presence and extent of facilitation, which does not depend on release, would have the same effect on the state of the synapse at the time of the second response regardless of the success or failure of the first response.

To evaluate whether depletion of vesicles causes short-term depression at excitatory hippocampal synapses we considered the success rate for the second response based on the success or failure of the first response using stimuli with a fixed IPI of 50 ms (figure 5(A)). At no synapse studied was there a significant impact of first response success on second response success (Fisher's exact test p -values 0.53, 0.29, 0.10, 0.60 and 1.00, respectively, for synapses from top to bottom). As the failure rate in response to the first presynaptic AP was generally higher, we then considered this rate based on the success or failure of the second pulse as a test for a fluctuating synaptic state (Volynski *et al* 2006) that might affect the release dependence result (figure 5(A); Fisher's exact test p -values the same, as second pulse success/failure given first pulse success/failure is the transposed matrix of first pulse success/failure given second pulse success/failure). The absence of such a relationship is consistent with fluctuations in the synaptic state having no effect on responses at the 50 ms IPI used here. Despite our efforts to limit release to at most a few sites, it was still possible that several release sites were release-capable, each having a low p_r . If microperfusion PSCs consist of multiple quanta, the synaptic current due to a single quantum would need to be considerably smaller than spontaneous synaptic currents recorded in the absence of APs, which have sizes similar to those of evoked microperfusion waveforms (figure 3). In the

case of several release sites each contributing very small quantal currents, even if depletion affected individual sites, the large number of sites could mask the effect because success could frequently be followed by success due to release from one of the other sites; if a synaptic terminal within the microperfusion region contains n identically behaving release sites, the probability of all sites failing is $(1 - p_{\text{site}})^n$. However, if depletion influences responses, the second response should still tend to be smaller when the first response succeeds. Hence, we examined the size distribution of second responses based on whether the first response succeeded or failed (figure 5(B)). Kolmogorov–Smirnov (K-S) tests of the distributions showed no evidence at five synapses tested that depletion played any role in determining the size of the subsequent responses as the sample distributions $\text{PSC}_{2,\text{PSC1 succeeded}}$ and $\text{PSC}_{2,\text{PSC1 failed}}$ were similar: the probabilities of observing at least this degree of similarity if samples were drawn from the same underlying distribution were 0.60, 0.25, 0.94, 0.95 and 0.51 at the five synapses studied. To test for an influence of the state of the synapse on these responses, we also considered PSC_1 based on whether PSC_2 succeeded or failed on that trial (figure 5(C)). The probabilities of differences for $\text{PSC}_{1,\text{PSC2 succeeds}}$ and $\text{PSC}_{1,\text{PSC2 fails}}$ taking at least the magnitude observed, in the event of a single underlying distribution, were 0.23, 0.28, 0.27, 0.07 and 0.53 at the 5 synapses, providing no evidence for fluctuations affecting amplitude. To consider the effect of depletion across a range of intervals, in experiments using other neurons we varied the IPI after the first AP to be either 25, 50, 100, 200 or 400 ms and compared first and second PSC values relative to the average response following the first and second AP in the train, respectively, at that synapse (figure 5(D)). If depletion influences synaptic responses, large PSC_1 responses would tend to be followed by small PSC_2 , and small PSC_1 by large PSC_2 , but no clear trend was observed at any of these intervals (number of PSC pairs evaluated for 25, 50, 100, 200 and 400 ms, respectively: 29, 84, 29, 28, 28).

We next sought to determine the extent to which PSCs contain a record of the timings of recent APs at the synapse. For this purpose we used stimulation trains containing a variable IPI followed by a fixed IPI. Using four-pulse trains with a first IPI of 50 ms, we compared synaptic responses after the last IPI (50 ms) based on the duration of the penultimate, variable IPI (50 or 400 ms, figure 6). Some asynchronous release is observed because these are responses to the final pulse in the train; note that asynchronous events, generally taken to be quantal, are similar in size to the phasic microperfusion PSCs. Whereas the varied interval influenced responses that immediately followed it (short-term dynamics; not shown), one AP later, no visually detectable difference between the waveforms of the two groups was apparent. The cumulative distributions of charge at each synapse also showed no difference: the K-S test p -value for the response to the final AP grouped by penultimate IPI at the synapse in figure 6(A) was 0.19 and at the synapse in figure 6(B) was 0.93. Across the remaining synapses, p -values were 0.60, 0.52 and 0.14.

Modeling has supported the idea that adjacent boutons along the axon function independently (Franks *et al* 2002), but to our knowledge this has not been tested directly. In rare cases (three synapses, one case sufficient duration for analysis), synapses formed by the presynaptic cell onto both the presynaptic cell itself ('autapses'; Van der Loos and Glaser 1972) and the postsynaptic neuron were present within the microperfusion stream. This

presented an opportunity to gauge the activity of the two sites simultaneously. When the presynaptic cell was stimulated in brief trains, successes were observed in the autaptic and synaptic currents, indicating release from neighboring focally perfused sites on the axon of the presynaptic neuron (figure 7). The presence of the synapse and autapse within the stream provides information on whether failure of AP propagation at branch points ('branch failure') contributes to responses to brief trains. AP initiation was confirmed by observation of presynaptic action current for all events reported in the study. In this recording from a microperfused synapse and autapse, although the synapse failed after the 4th AP on 57% of trials, both the synapse and autapse failed on only 14% of trials, consistent with the bulk of failures being due to failure of exocytosis rather than of AP propagation. Minimal or zero contribution of branch failure is necessary for independence of nearby synapses, but not sufficient. To address the independence of sites, we compared the responses of the autapse and synapse on individual trials, observing that failures and successes did not tend to co-occur. To quantify this, we calculated the co-occurrence relative to that expected by chance given the success rates of each synapse. Based on their individual probabilities of success after the first AP, the autapse and synapse would have been predicted to succeed on the same trial by chance 2.67 times during the experiment, and they did succeed on the same trial 3 times (Agresti–Coull 95% CI 0.91–7.49). After the fourth AP, they were expected to succeed on the same trial 6.43 times by chance, and they did so 6 times (Agresti–Coull 95% CI 2.88–10.56). The overall correlation coefficient for synapse and autapse success/failure was 0.03 (probability of observing a correlation coefficient of this magnitude or greater if no true correlation $p > 0.75$). These results are consistent with independent function of the nearby sites.

Discussion

Advantages of the method for investigations of synaptic plasticity

Localized perfusion was introduced by Katz and Miledi (1965) and refined by Maconochie and Knight (1989), Streit and Lux (1989), Ogata and Tatebayashi (1991), Kraszewski and Grantyn (1992), Bekkers and Stevens (1995), Veselovsky *et al* (1996), Engert and Bonhoeffer (1997) and others. The microperfusion method presented here, a member of this group of techniques, permits multi-hour stable recordings of AP-evoked transmission between identified pairs of neurons with responses consistent with activity of single release sites on individual trials. By titrating culture conditions to achieve roughly 5–15 neurons per glial island, neurons receive less dense projections from each individual presynaptic cell than expected in widely used culture conditions having fewer neurons per island, making it easier to limit activity to few presynaptic boutons. Given the spacing between *en passant* boutons along axons of cultured hippocampal neurons during the age range used for experiments (Liu and Tsien 1995, Forti *et al* 1997, Darcy *et al* 2006), the microperfusion stream used in these studies likely permits at most 2–3 boutons from the presynaptic neuron to be active during an experiment, even with facilitation. Absent this, early in trains, responses are likely from the best-perfused bouton and monoquantal. The reduction of PSC magnitude relative to standard perfusion was generally from 10- to 50-fold. Because both pre- and postsynaptic neurons are patched, there is less non-biological current flow in the vicinity of synapses than with extracellular stimulation, and the experimenter can confirm

that each AP was successfully evoked by visualizing the action current. Many of the processes underlying short-term dynamics are likely to depend on the steps between AP firing and exocytosis, so evoking release by triggering APs in the presynaptic soma provides a suitable model for studies of synaptic transmission. With microperfusion, the experimenter knows the transmitter phenotype and cell of origin for each bouton. Single-neuron staining can be used to label individual boutons for positioning of the microperfusion system. As the site of synaptic activity is fixed at one location in the postsynaptic neuron's dendritic tree throughout the experiment, the imperfect space-clamp inherent in voltage-clamp is limited to a single electrotonic distance, avoiding artifact that would occur if sites at other distances became active. Perforated patch-clamp permits stable recording for several hours from synaptically connected pairs of neurons with minimal disruption of intracellular contents. Because of the small contribution of Ca^{2+} flux to PSCs at the postsynaptic holding potentials used for most synaptic studies (Skeberdis *et al* 2006), any reduction in $[\text{Ca}^{2+}]$ due to a mixture of global perfusate into the microperfusion stream is expected to have little measurable effect on PSCs. The elimination of polysynaptic activity with focal perfusion removes a major potential confound to experiments in neural culture. When reducing the number of active synapses by lowering global calcium, there is no summative effect on PSC waveforms across multiple APs as would be expected for a competitive antagonist such as CNQX. Together, these elements make the system well suited for studies of AP-evoked release and short- and long-term plasticity at individual synaptic contacts. In addition, as the accessible geometry of the culture configuration promotes rapid diffusion, single bouton synaptic current appears or disappears rapidly when the positive pressure is turned on or off (figure 3(A)). Thus, it is likely that studies requiring application and removal of local calcium or other substances on fine time scales would also be feasible.

Waveform variability

Waveforms recorded in the microperfusion configuration vary in size at each synapse (figure 3). Intrasite variability is also seen in other studies of individual synapses (for example, Frerking *et al* 1995, Liu and Tsien 1995, Forti *et al* 1997, O'Connor *et al* 2005, Perea and Araque 2007), indicating that this may be a fundamental feature of single-site transmission. Interestingly, amplitude distributions from microperfusion tend to be similar to or smaller than those of spontaneous miniature PSCs (figure 3(C)), and even with microperfusion directly on the postsynaptic soma very small events are still observed (supplementary figure S4 available at stacks.iop.org/JNE/9/036014/mmedia). The larger events seen in TTX may implicate coordinated vesicle release in the absence of APs (Llano *et al* 2000, Frerking *et al* 1997). If large spontaneous synaptic currents involve such coordination, the size disparity suggests that the region of coordination exceeds the region of microperfusion.

Depletion as a cause of short-term depression

The assumption that vesicle exocytosis at a site temporarily reduces the likelihood of subsequent release underlies widely used interpretations of the paired-pulse ratio as an indicator of the locus of long-term potentiation. If depletion influences synaptic currents, the response to the second of a pair of stimuli should be smaller following a successful release than following a failure. However, at the SC-CA1 synapse in slice, Cabezas and Buño

(2006) demonstrated that the potency of a polyquantal PSC is unaffected by the success or failure of an AP occurring 50 ms earlier in evoking synaptic current. Also calling into question the importance of depletion for short-term depression, in cultured hippocampal neurons the paired-pulse ratio at 45 ms is the same before and after a massive exocytosis provoked with a hyperosmotic solution (Sullivan 2007). In this study, we found that neither the presence nor the size of responses to a second AP are influenced by the success or failure of release following the first AP (figure 5), further indicating that vesicle depletion is not a major influence on short-term plasticity at these synapses at the IPIs used. However, depletion could still contribute if replenishment of the readily-releasable vesicle pool happens more rapidly than could be detected with the IPIs used in this study (Hanse and Gustafsson 2001b). Refilling of the immediately releasable pool requires approximately 20 ms (Stevens and Wang 1995) to 200 ms (Hanse and Gustafsson 2001a). Our IPIs ranged from 25 to 400 ms and so would likely have been sufficient for such refilling. Accurate data on depletion from paired-pulse ratio for assessment of locus of synaptic plasticity may require briefer IPIs than are generally used.

History dependence

Whatever the mechanisms of short-term plasticity at a given synapse, the processes set in motion when an AP invades the synaptic terminal, leading to facilitation or depression of the subsequent response, could have cumulative effects across multiple responses that would reflect the number and timing of recent APs. The amount of information on the timing of recent presynaptic APs possibly conveyed by single PSCs due to synaptic dynamics has been shown to be considerable in theoretical studies (Maass and Markram 2002), such that PSC fluctuations could contain information about the durations of as many as eight preceding IPIs (Fuhrmann *et al* 2002). However, given the stochastic nature of synaptic function, the extent to which information about history can be extracted from short-term dynamics at synapses consisting of few release sites is unclear. The larger the number of synaptic contacts formed by the presynaptic neuron onto the postsynaptic, the more deterministic their collective behavior, but in many brain regions, the synapse consists of few contacts (Deuchars and Thomson 1996) or a single one (Gulyás *et al* 1993). The limited information on recent activity history present in the responses of individual hippocampal synaptic contacts (figure 6) indicates that when few contacts are active there is no clear mechanism by which the postsynaptic cell could 'read out' the recent history of activity in the presynaptic neuron from a single PSC. The more deterministic responses of multi-bouton synapses could thus potentially allow for the more robust transfer of information on recent activity history to the postsynaptic neuron in single PSCs, a consideration that may influence the coding principles at work in different neural circuits.

Independent function of nearby synapses

As an AP progresses down the axon, nearby synaptic sites might be expected to function with some degree of correlation to one another; the metabolic state of nearby regions is likely to be similar, and autocrine and paracrine influences are likely to overlap. Indeed, release probabilities are locally correlated at sites along the same axon or dendrite (Murthy *et al* 1997, Branco *et al* 2008). Moreover, the activity at one synaptic terminal on the axon influences subsequent activity at nearby terminals through overspill of neurotransmitter

(Vogt and Nicoll 1999), which could serve to standardize release probability at nearby sites. The degree of crosstalk will depend on the glial ultrastructure (Piet *et al* 2004), which physically constrains diffusion and further influences the rate of transmitter removal from the cleft through transporter action. However, in the preparation used here, astrocytes are seldom found near synapses (Forti *et al* 1997). Moreover, hippocampal synapses under microperfusion generally have relatively low release probability, and the stimulation trains used are brief, so overspill is unlikely to play a large role; postsynaptic evidence of overspill in the form of bowed waveforms was not observed.

Analysis of one case where both autapse and synapse were present within the microperfusion stream suggested that at nearby release sites, success or failure of release after each AP is uncorrelated (figure 7). Given the potential for differential short-term dynamics from a single presynaptic neuron onto different postsynaptic targets (Markram *et al* 1998), independence of nearby synapses is expected to influence the transfer of information between neurons (Franks *et al* 2002); information storage and transmission capacity may be maximized when synapses operate independently (Barbour 2001). AP propagation failure (Debanne *et al* 1997) would increase the correlation of success and failure at local sites, and thus is unlikely to contribute during brief trains of APs as used here. At inhibitory synapses, the independence of synaptic function is influenced by the number of stimuli or the anatomical spacing of sites (Overstreet and Westbrook 2003). Thus, developmental change, AP timing or neurotransmitter phenotype could influence the independence of adjacent contacts, altering information transfer by the presynaptic neuron. While the present study suggests that excitatory synapses located within a few μm of one another on the axon function independently at low firing rates, further studies are needed to assess the extent to which release sites within a single terminal (Schikorski and Stevens 1997) operate in a correlated fashion (Ran *et al* 2009).

Suitability for studies of long-term potentiation

The similarity of the waveforms recorded using the microperfusion system to naturally small waveforms suggests that glutamate dwell time in the synaptic cleft is comparable in the two conditions (figure 3(D)). This is consistent with minimal effect of the liquid microperfusion current within the cleft itself, so transsynaptic diffusion of possible retrograde messengers is unlikely to be disturbed. Non-disruption of diffusional processes is a precondition to the use of the system for studies of activity-dependent synaptic plasticity over hours (long-term plasticity).

The method presented here permits alteration of the environment of a circumscribed portion of individual cells without removal from the position they take during growth. It is suitable for any adherent cell type and does not require culturing in isolation or in a specialized environment, which can compromise viability or alter phenotype and may also be prohibitively expensive or difficult. The microperfused location can be chosen and modified during the course of the experiment. The extent of the microperfusion environment is finely controllable. Its stability can be confirmed visually and noninvasively monitored during experiments with acoustic information. The modular elements of the method can be combined in the manner best suited to answering the question at hand and are

straightforward to implement. It is our hope that investigations of the cellular and molecular bases of synaptic function and plasticity will benefit from its use.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The microperfusion system. (A and B) Photomicrograph and schema of the system. The axon of the presynaptic neuron meets a dendrite of the postsynaptic neuron in the middle of the frame. The positive pressure microperfusion tip (right) is apposed to the neurites. It delivers normocalcemic solution to the cells. The solution is then removed by the negative pressure tip (left). Scale bar 25 μm . (C) The microperfusion flow, not visible under standard experimental conditions. (D) Reduction of the number of active synapses with microperfusion. Left: overlay of two trials in the same experiment, one under standard perfusion and one under microperfusion. Right: close-up of the microperfusion trial showing the area overlaid with the gray box on the left.

Figure 2.

Siphon-suction junction used to maintain stable low negative pressure and produce the acoustic signal, and use of the acoustic signal to monitor microperfusion flux. (A) The mounting and configuration of the siphon-suction junction (right) and the microphone (left). The junction housing is mounted on a vertical track finely adjustable (yellow knob) to regulate height. The gap between the siphon and suction tips is exposed to the air through the housing aperture. The microphone is mounted on a horizontal track that is adjusted to bring the microphone tip closer to the aperture. (B) Detail of the siphon-suction junction

(cross-sectional view). The siphon outflow tubing (green) is connected to a metal cylinder that enters the housing from above and is sealed by a rubber o-ring (orange). A similar arrangement exists for suction from the bottom of the housing apparatus. (C) Recordings at 15 min intervals show stability over time. (D) Audio traces recorded at 40 s intervals. An induced block occurred between trials 124 and 125, changing the signal. (E) The negative pressure of the siphon, regulated by varying the height of the siphon-suction junction, alters flux as reflected in the signal. Signal amplitude is in arbitrary units but the same scale is used in all panels. Note that in (E), a characteristic pattern is present for each siphon pressure, but signal amplitude reflects the distance of the microphone from the aperture and is not directly comparable across siphon heights.

Figure 3.

Validation of the microperfusion system. (A) Local application of normocalcemic solution is necessary for evoked synaptic transmission. Three consecutive trials are shown, earliest at bottom. Positive pressure to the microperfusion tip was stopped between the first and the second trial, and then resumed following the second trial. Inset shows average response to trains with briefer IPI₂ (50 ms; top) and longer IPI₂ (400 ms; bottom); trains with briefer IPI₂ were at least as likely at this synapse to result in successful transmission. (B) Functional stability of the microperfusion system tested at five synapses. Responses are to the first

presynaptic AP in each train unless those responses were essentially all synaptic failures, in which case the second response in each train is shown. Filled symbols reflect successful transmission; hollow symbols reflect failure of synaptic current. Dotted line shows a linear fit. The data in the last case were recorded with the positive, but not the negative, component of the microperfusion system in place. (C) PSC waveforms from four different classes of 'quantal' experiment. Left column, detected synaptic current waveforms (colored traces, individual trials; black trace, average). Right column, fits to the synaptic current waveforms. For fitting, see the methods section. Top row: evoked events from a weak connection recorded in a normocalcemic solution. Second row: evoked events in microperfusion configuration. Third row: events evoked by a single 10 s application of a hyperosmotic solution in the microperfusion configuration. Fourth row: spontaneous events recorded over 810 s in 1.5 μ M tetrodotoxin, 10 μ M bicuculline methiodide. (D) Average waveforms (top two panels) and leak-corrected, latency-corrected fits (bottom two panels) for the four conditions shown in (C). In the second and fourth panels, traces are scaled to the same peak amplitude.

Figure 4.

Latency at individual synapses. (A) Method for measuring latency. Left: detection of the onset of presynaptic action current (green circle), taken as time zero. Center: the artifact in the postsynaptic membrane current recording due to the end of the presynaptic stimulation (cyan) is removed to eliminate a potential source of error. Right: membrane current (black) and detection criterion (red) with threshold (blue). Crossing of threshold is used to indicate successful release, and the time of the peak in the detection criterion (vertical arrow) is taken as the event onset time. (B) Latency measurements at individual synapses. Cumulative

histograms of latencies following the first (red), second (green), third (blue) and if applicable fourth (black) pulses in a train, with mean values in bar graphs. Error bars show SEM. A double response was seen in one case (first column, third down), leading to bimodal appearance of cumulative histograms. An example waveform from this site and the detection criterion are shown in the inset.

Figure 5.

Depletion does not influence paired-pulse responses at individual synaptic contacts. (A) Hollow bars show mean and error (Agresti–Coull binomial confidence interval) proportion of PSC₂ successes depending on whether PSC₁ succeeded (left) or failed (right) at five synapses. Filled bars show the success proportion for PSC₁, depending on whether PSC₂ in that trial succeeded (left) or failed (right). The success of either PSC does not predict that of the other. (A through C 50 ms IPI). (B) Cumulative histogram of the size (pA) of PSC₂ depending on the success (solid) or failure (dashed) of PSC₁ each trial. (C) Cumulative histogram of the size of PSC₁ separated by the success (solid) or failure (dashed) of PSC₂ each trial. (D) Size of PSC₁ versus size of PSC₂ by IPI at seven to ten synapses over five possible IPIs, 25, 50, 100, 200 and 400 ms. PSC₁ values are normalized to $\langle PSC_1 \rangle$, PSC₂ to $\langle PSC_2 \rangle$. Each color represents a synapse, each dot represents a pair of values and each line represents a linear fit to the value pairs for that synapse. Depletion is expected to result in smaller PSC₂ following larger PSC₁; this is not observed.

Figure 6.

The IPI before the most recent IPI does not affect the synaptic response. (A) Left: waveforms elicited by the fourth AP in a train of four when the IPIs were 50, 50, 50 ms. Because this is the final pulse in the train asynchronous release is evident. Middle: waveforms elicited by the fourth AP when the IPIs were 50, 400, 50 ms. Right: cumulative histogram of the charge values for the two stimulus trains. Responses to the 50, 400, 50 trains are shown with the dotted line. (B) As (A) but for a different synapse. Note that asynchronous release events, which represent ‘quanta’, are comparable in size to many of the evoked waveforms.

Figure 7.

Synapses adjacent on the axon may function independently. (A) Autaptic (red) and synaptic (blue) currents both arise within the focally perfused region, indicating proximity on the axon. (B) Success of autapse alone. (C) Success of synapse alone. (D) Failure of both autapse and synapse. Inset: histogram of latencies of the two classes of events. Successful synaptic PSCs occurred an average of 1.1 ms earlier than successful autaptic PSCs; this is likely caused by delay in event detection due to the action current in the autaptic trace.