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Neuronal activity drives matching of pre- and post-synaptic function during synapse maturation

Louise Kay, Lawrence Humphreys, Britta J. Eickholt and Juan Burrone

MRC Centre for Developmental Neurobiology, King's College London, 4th Floor,
New Hunt's House, Guy's Campus, London SE1 1UL, UK.

The structure and function of presynaptic and postsynaptic compartments varies dramatically within neurons, but little is known about how they are functionally arranged with respect to each other. In rat hippocampal neurons we found that, although they are structurally correlated from the early moments of formation, synapses only gradually become functionally matched, and that this process is dependent on ongoing electrical activity.

Postsynaptic spines are a prime example of structural diversity, showing a variety of morphologies that include variations in spine head volume of up to an order of magnitude [1]. This structural diversity appears to be tied to its function: larger spines show larger postsynaptic responses than smaller ones [2]. Similarly, the presynaptic terminal displays a wide range of structurally diverse boutons, with large variations in both total vesicle number and number of docked vesicles at the active zone. Indeed, the latter is thought to predict release probability, which in turn can vary by up to 2 orders of magnitude within boutons of a single axon [3-4]. Is this large repertoire of distinct pre- and postsynaptic compartments structurally and functionally matched? In other words, are large spines with many postsynaptic receptors contacted by large boutons with high release probability? Observations at the ultrastructural level suggest that there is indeed a high degree of correlation between bouton volume, total vesicle number, active zone area, postsynaptic density area and spine volume [5-8]. Whilst concrete experimental evidence in support of a functional correlation is lacking, indirect evidence suggests that presynaptic release probability is *not* correlated with postsynaptic surface GluR2 levels, unless neurons are grown in a medium conducive to high levels of activity [9].

We set out to study the structure and function of individual synapses in dissociated hippocampal neurons, to establish whether any correlation exists between pre- and post-synaptic compartments. Immunostaining of synaptic proteins in mature neurons (14 days *in vitro*; div) showed a strong correlation between the fluorescence intensity of the vesicular glutamate transporter (vGlut-1) puncta and their corresponding postsynaptic density-95 (PSD-95) or GluR2 puncta (**Fig. 1**). This correlation was already apparent at earlier time points, as early as 7 div, a time when synapses are just beginning to form in our hippocampal cultures. In addition, blocking action potentials in the entire network, from 10 to 14 div, by incubating neurons with the voltage-gated sodium channel antagonist tetrodotoxin (TTX), had no effect on this correlation (**Fig. 1**).

Next, we asked if presynaptic and postsynaptic compartments are also functionally correlated. The size of the recycling pool of vesicles has previously been shown to correlate well with the number of docked vesicles at the active zone [3], which in turn is thought to determine release probability. Therefore, we performed live labelling of presynaptic vesicles using the fluorescent membrane dye FM4-64 in response to a saturating stimulus that mobilizes the entire recycling pool (see methods and **supplementary Fig. 1**). To simultaneously assess post-synaptic function at individual synapses we recorded the postsynaptic currents elicited by uncaging ‘caged-glutamate’ at the corresponding postsynaptic spine (**Fig. 2a**). We recorded AMPA-type glutamate receptor currents (uEPSCs) from postsynaptic neurons that were either expressing EGFP (in a sparsely expressing neuronal culture, **Fig. 2b**) or were filled with green fluorescent alexa dye (alexa-488) from the patch pipette (**Fig. 2c**). This allowed us to visualize the spines along the dendrites of individual neurons and to position the uncaging laser (405 nm) at a site where a presynaptic FM4-64 puncta was present (**Fig. 2b-c**). In this way, we could correlate the fluorescence intensity of a presynaptic terminal to the amplitude of postsynaptic glutamatergic currents at the corresponding spine (**Fig. 2e-g**). Brief (0.5 and 1 ms) pulses of 405 nm light were delivered at a single point and postsynaptic currents were measured at the soma (**Fig. 2c**). Increasing the duration of the laser pulse elicited larger postsynaptic responses, with similar activation kinetics (**Supplementary Fig. 2**). We found that a 0.5 ms pulse was similar in size to a miniature postsynaptic current (mEPSC) and a 1 ms pulse was roughly equivalent to 2-3 quantal events (**Supplementary Fig. 2**). We

decided to use these two stimulus durations for the remainder of our experiments. Importantly, we also found that the spatial resolution of this technique was sufficiently high to allow unequivocal stimulation of individual spines, without contamination from neighbouring ones (**Supplementary Fig. 3**). Figure 2c shows a neuron in which we measured the responses to uncaging pulses at spines that showed a clearly labelled FM4-64 punctum. We found that individual cells (an example of which is shown as the green circles in Fig. 2f), as well as pooled data from multiple cells (black circles in **Fig. 2f**, from 11 cells and 103 synapses) showed a strong correlation between the amplitude of the postsynaptic response (for both 0.5 and 1 ms pulses) and the corresponding presynaptic FM4-64 fluorescence intensity. A similar correlation was also observed between the total charge of the postsynaptic response and FM4-64 intensity (data not shown). In agreement with the pooled data from many cells, we also found that around 70 % of single neurons showed a significant correlation (60 % for a 0.5 ms pulse and 80 % for a 1 ms pulse; only neurons where more than 8 synapses had been assessed were included in this analysis). Many variables can influence postsynaptic recordings at the soma, such as distance of synapses from the soma and the intrinsic properties of the neuron. We found no correlation existed between any of these parameters (**Supplementary Fig. 4**) or other possible experimental variables that could potentially affect our data (**Supplementary Fig. 5**). We were curious to establish if this form of ‘synaptic matching’ found in mature networks was also present earlier on in development. Most synapses in our system form between day 6 and day 11 *in vitro* [10]. We therefore focused on div 10, a time period that is characterized by the presence of many newly formed synapses. Here, no correlation or only a weak correlation existed between postsynaptic responses to focal uncaging and presynaptic FM4-64 fluorescence intensity (**Fig. 2e**; 9 cells, 78 synapses). This significant decrease in the correlation for the pooled data was mirrored by a complete absence of any correlation at the single cell level (0 % of cells for a 0.5 ms pulse or a 1 ms pulse). However, we were concerned that the smaller amplitude uEPSCs recorded at 10 div (**Supplementary Fig. 2c**) was somehow masking the correlation observed at 14 div. We therefore also performed uncaging experiments using short stimuli (0.25 ms) at 14 div that elicited uEPSCs of similar amplitude to those measured at 10 div (**Supplementary Fig. 2c**). We found a significant correlation existed even for short duration pulses at 14 div (**Supplementary Fig. 6**), further strengthening our conclusions. Finally, we tested

whether the emerging correlation in synaptic function needed activity to develop. Neurons were incubated in TTX during the period of synapse maturation (10 to 14 div), to abolish action potentials in the entire network. We found that the functional correlation normally present in mature neurons, was now absent in both pooled data and individual neurons (a significant correlation was observed in only 33 % of neurons for either a 0.5 or 1 ms pulse), although the amplitude of both pre- and postsynaptic measurements were of comparable size to 14 div control neurons (**Fig. 2g**; 11 cells, 73 synapses; mean FM4-64 intensity (A.U.) for Control: 172.4 +/- 11.8 and TTX: 283 +/- 18.15; uEPSC, 0.5 ms (pA) for Control: -55.37 +/-2.6 and TTX: -38.23 +/- 2.02; uEPSC, 1 ms (pA) for Control: -93.71 +/- 4.5 and TTX: -73.1 +/- 4.2).

Together, our findings suggest that pre- and postsynaptic *structures* are matched early on in development and that this correlation is insensitive to manipulations that abolish neuronal activity. However, *functional* measurements are originally not well correlated, but instead emerge during synapse maturation in an activity-dependent manner. This form of synaptic matching provides important evidence for cross-talk at the synapse and further suggests that active mechanisms exist that control the strength of both pre- and post-synaptic compartments in unison, in agreement with previous findings [9].

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Author information

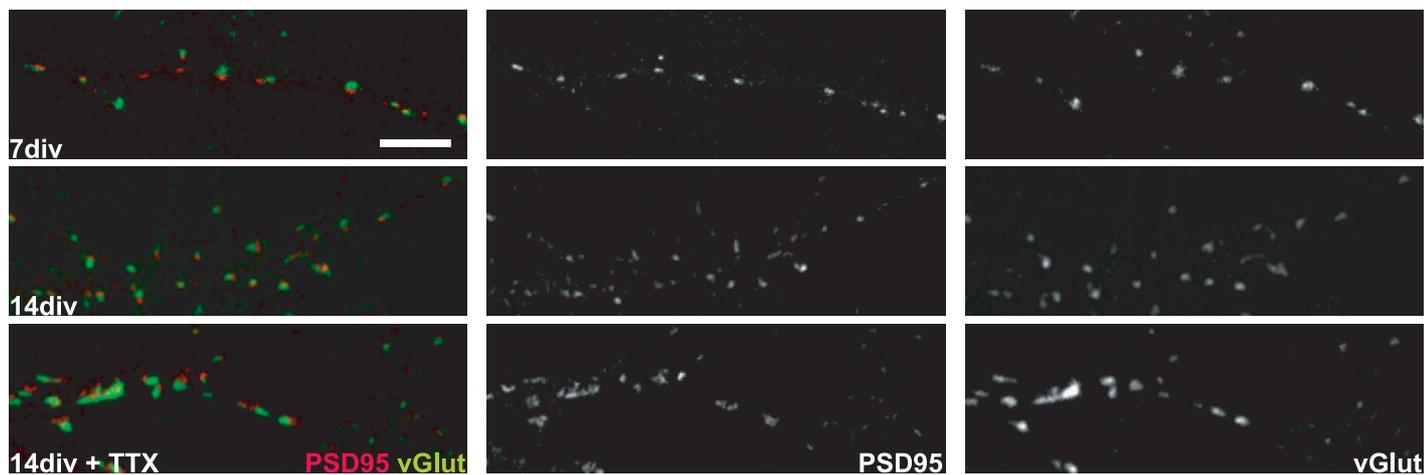
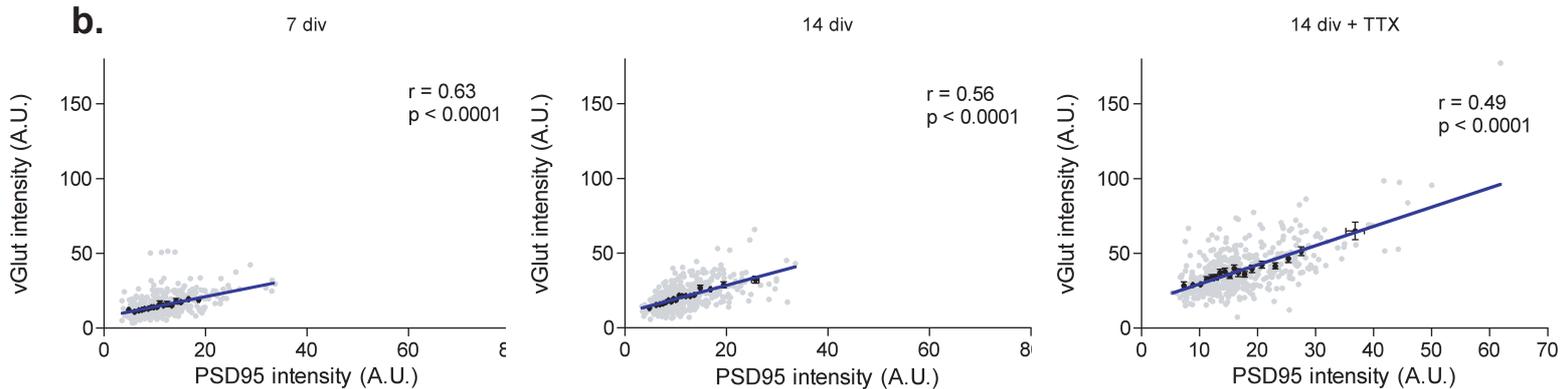
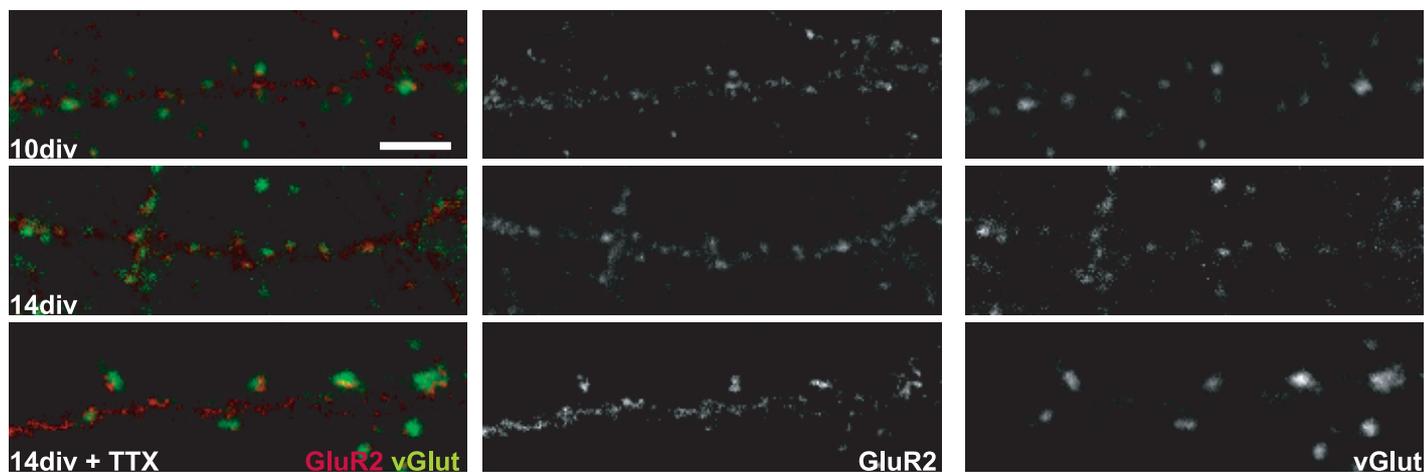
Correspondence and requests for materials should be addressed to J.B. (juan.burrone@kcl.ac.uk).

Figure 1. Matching of synaptic structure in young and mature neurons. (a)

Example images of hippocampal neurons stained with vGluT (green) and PSD-95 (red) at two different developmental stages (7 and 14 div), for control neurons and neurons treated with TTX from 7 div. (b) Plot of fluorescence intensity for vGluT staining as a function of PSD-95 staining per synapse (gray points) and grouped in bins of 25 (black circles) for 10 cells and 450 synapses. Mean fluorescence intensity at 7 div: vGluT: 15.44 +/- 0.32, PSD95: 11.64 +/- 0.23; at 14 div: vGluT: 20.80 +/- 0.39, PSD95: 11.57 +/- 0.24; at 14 div after TTX treatment: vGluT: 38.72 +/- 0.71, PSD95: 17.13 +/- 0.35. (c) Example images of neurons stained with vGluT (green) and GluR2 (red) at two different developmental stages (10 and 14 div), for control neurons and neurons treated with TTX from 10 div. Mean fluorescence intensity at 10 div: vGluT: 422.1 +/- 9.49, GluR2: 184.6 +/- 4.60; at 14 div: vGluT: 442.0 +/- 7.38, GluR2: 218.0 +/- 3.58; at 14 div after TTX treatment: vGluT: 597.2 +/- 8.91, GluR2: 319.4 +/- 5.18.

(b) Plot of fluorescence intensity for vGluT staining as a function of GluR2 staining per synapse (gray points) and grouped in bins of 25 (black circles) for 9 cells and ≥ 780 synapses.

Figure 2. Activity-dependent matching of synaptic function during synapse maturation. (a) Schematic diagram of a green spine and its corresponding presynaptic terminal containing FM4-64-labeled vesicles (red). The blue circle depicts the site of glutamate uncaging. (b) Examples of dendrites and spines expressing EGFP (green) and FM4-64 labeled presynaptic terminals (red). (c) Confocal image of an Alexa-488 filled neuron (green) with presynaptic boutons labelled using FM4-64 (red). The responses to glutamate uncaging at individual synapses recorded in voltage-clamp, held at -70 mV, are shown in white. The blue dots show the uncaging site. (d). Examples of currents evoked in response to 0.5 ms and 1 ms glutamate uncaging (on arrow head) at a single synapse in 10div and 14div neurons and in 14div neurons following 4 days of TTX treatment. (e-g) The size of the response to 0.5ms and 1ms glutamate uncaging was compared to the intensity of FM4-64 labelling at each synapse. In 10 div neurons there is weak or no correlation between presynaptic FM4-64 intensity and the amplitude of post-synaptic currents (0.5 ms uncaging pulse: $r = 0.25$, $p = 0.03$; 1 ms uncaging pulse: $r = 0.34$, $p = 0.001$; e). In 14 div neurons synapses become functionally matched and a correlation is apparent (0.5 ms uncaging pulse: $r = 0.44$, $p < 0.0001$; 1 ms uncaging pulse: $r = 0.45$, $p < 0.0001$; d). The process of synapse matching is impeded by using TTX to block action potentials for 4 days (0.5 ms uncaging pulse: $r = 0.22$, $p = 0.06$; 1 ms uncaging pulse: $r = 0.18$, $p = 0.11$; e).

a.**b.****c.****d.**