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https://doi.org/10.1523/ENEURO.0540-20.2021

Cite as: eNeuro 2021; 10.1523/ENEURO.0540-20.2021

Received: 12 December 2020
Revised: 20 September 2021
Accepted: 24 September 2021

This Early Release article has been peer-reviewed and accepted, but has not been through the composition and copyediting processes. The final version may differ slightly in style or formatting and will contain links to any extended data.

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Selective increase of correlated activity in Arc-positive neurons after chemically induced long-term potentiation in cultured hippocampal neurons

Network connectivity and Arc engrams

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Number of Figures: 10

Number of Tables: 1

Number of Multimedia: None

Number of words for Abstract: 225

Number of words for Significance Statement: 119

Number of words for Introduction: 724

Number of words for Discussion: 2485

Acknowledgements: None

Conflict of Interest: The authors declare no competing financial interests.

Funding sources: MOH National Medical Research Council Grant OFIRG16May052.
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ABSTRACT

The activity-dependent expression of immediate-early genes (IEGs) has been utilised to label memory traces. However, their roles in engram specification are incompletely understood. Outstanding questions remain as to whether expression of IEGs can interplay with network properties such as functional connectivity and also if neurons expressing different IEGs are functionally distinct. In order to connect IEG expression at the cellular level with changes in functional-connectivity, we investigated the expression of 2 IEGs, Arc and c-Fos, in cultured hippocampal neurons. Primary neuronal cultures were treated with a chemical cocktail (4-aminopyridine, bicuculline, and forskolin) to increase neuronal activity, IEG expression, and induce chemical long-term potentiation. Neuronal firing is assayed by intracellular calcium imaging using GCaMP6m and expression of IEGs is assessed by immunofluorescence staining. We noted an emergent network property of refinement in network activity, characterized by a global downregulation of correlated activity, together with an increase in correlated activity between subsets of specific neurons. Subsequently, we show that Arc expression correlates with the effects of refinement, as the increase in correlated activity occurs specifically between Arc-positive neurons. The expression patterns of the IEGs c-Fos and Arc strongly overlap, but Arc was more selectively expressed than c-Fos. A subpopulation of neurons positive for both Arc and c-Fos shows increased correlated activity, while correlated firing between Arc+/cFos- neurons is reduced. Our results relate neuronal activity-dependent expression of the IEGs Arc and c-Fos on the individual cellular level to changes in correlated activity of the neuronal network.

Keywords: Arc, IEG, activity-dependent, c-Fos, network LTP, functional connectivity

SIGNIFICANCE

Establishing a stable long-lasting memory requires neuronal network-level changes in connection strengths in a subset of neurons, which together constitute a memory trace or engram. Two genes, c-Fos and Arc, have been implicated to play critical roles in the formation of the engram. They have been studied extensively at the cellular/molecular level, and have been used as markers of memory traces in mice. We have correlated Arc and c-Fos cellular expression with refinement of correlated neuronal activity following pharmacological activation of networks formed by cultured hippocampal neurons. Whereas there is a global loss of correlated activity, Arc-positive neurons show selectively increased correlated activity. Arc is more selectively expressed than c-Fos, but the two genes act together in encoding information about changes in correlated firing.

INTRODUCTION

Activity-driven neuronal plasticity is the basis for learning and memory. New tools in optogenetics and molecular biology have culminated in numerous studies in recent years which mark immediate-early gene (IEG)-expressing neurons as memory engrams (Liu 2012, Denny 2014, Tonegawa 2015). Transcriptional
programs activated upon neuronal stimulation are complex, with the involvement of a myriad of IEGs, their downstream effectors and the interplay between gene transcription and ongoing neuronal activity (Yap 2018). Although the activity-dependent expression of IEGs have been successfully utilised to label memory traces, their roles in engram specification is incompletely understood. For instance, although the expression profiles of IEGs within neuronal populations overlap significantly (Mahringer 2019), they are diverse and distinct (Gonzales 2019), with different IEGs performing distinct functions. Furthermore, it is known that within single neurons, there is a combinatorial expression of IEGs (Sheng 1995). This suggests that differential expression of IEGs can encode the specificity of the stimulus, which has been demonstrated in part using single-cell RNA-sequencing technology (Jaeger 2018). Arc/Arg3.1 (activity-regulated cytoskeleton-associated protein/activity-regulated gene 3.1) and c-Fos are two IEGs that are commonly used in memory engram labelling experiments (Kawashima 2014). Arc is an important player in synaptic plasticity and long-term memory consolidation (Plath 2006, Shepherd 2011, Nikolaienko 2018). c-Fos is another IEG whose increase in expression is commonly used as an indicator for neuronal activation. It was one of the first transcription factors whose induction was shown to be activity-dependent (Sheng 1990). It has been recently shown that IEGs Arc and c-Fos are differentially expressed within neuronal populations and their combinatorial expression is functionally linked to whether a memory trace is newly formed or reactivated (Jaeger 2018). This is due to the difference in the expression dynamics of the two IEGs, with c-Fos being transiently expressed after activation but Arc maintaining a high level of transcription hours after stimulus onset, specifically in the dentate granule neurons (Jaeger 2018). Further questions remain, however, as to whether expression of IEGs can interplay with patterns of correlated firing between neurons and if neurons expressing different IEGs are functionally distinct.

The fundamental cellular correlate of memory is synaptic plasticity, while engram formation is dependent on the connectivity of the neuronal network (Tonegawa 2015). Conventionally, studies of neuronal activity and IEG expression are mostly based on the single cell level whereas network activity is recorded on the neuronal population level. Thus there is a lack in linking IEG expression and changes in patterns of correlated activity changes on the network level. Similarly, studies on long-term potentiation (LTP) are extensive on the molecular, cellular and pathway level, but few studies have focused on the effects of LTP in altering patterns of correlated activity in a population of interconnected neurons. Among the various induction methods of LTP, direct chemical stimulation of specific biochemical processes to induce LTP can result in plasticity changes in large numbers of synapses from multiple neurons. NMDA receptor dependent LTP requires the coincidence of presynaptic glutamate release and postsynaptic depolarisation. Chemically, this can be accomplished by a variety of pharmacological agents. Among these, bicuculline, a GABA_A receptor antagonist has been shown to be able to induce bursts of action potential and elicit NMDA receptor-dependent calcium transients. When this is applied together with the presynaptic K^+ channel blocker 4-amino pyridine (4-AP), which enhances glutamate release, calcium transients in the neuronal nucleus increase from baseline levels (Hardingham 2001). This sustained elevation in nuclear calcium can increase the affinity of calmodulin binding to calcium and in turn result in maintained activation of Ca^{2+}/calmodulin-dependent enzymes that potentially facilitate transcription. The synergistic effects of these agents have been shown to be able to increase the endogenous expression of c-Fos (Hardingham 2001). Forskolin, an adenylyl cyclase activator, can effectively increase the level of cAMP levels and directly activate transcription and translation that is important for the expression and maintenance of protein synthesis-dependent late phase of LTP (Otmakhov 2004). It has also been shown that the translational repression of the IEG Arc is alleviated by application of forskolin, through activation of the cAMP-dependent protein kinase (PKA) pathway (Bloomer 2008). This combination of pharmacological agents, namely 4-AP, bicuculline and forskolin, has been used effectively to induce LTP in cultured hippocampal neurons in the study of Arc dynamics (Wee 2014, Oey 2015, Leung 2019).
We aim to relate neuronal activity-dependent expression of the IEGs Arc and c-Fos on the individual cellular level to changes in correlated activity in the cultured neuronal network. Changes in functional network connectivity can be studied systematically (Poli 2016), and these methods can be applied to cultured neuronal networks. We made use of intracellular calcium imaging (GCaMP6m) as a proxy measurement for neuronal activity changes. Expression of IEGs Arc and c-Fos is assessed by immunofluorescence staining and is subsequently matched with changes in correlated activity in specific subpopulations of neurons.

MATERIALS AND METHODS

Neuronal Cell Culture. Hippocampi from embryonic day 18 Sprague-Dawley rats of either sex was dissected in ice-cold Hanks Balanced Salt Solution (HBSS) and digested using a papain dissociation system (Worthington Biochemical Corporation). The density of the network was determined by a seeding density of 1.5 x 10^5 cells/cm². Neurons were plated on glass-bottom culture dishes (MatTek, Ashland, MA) that had been coated with poly-D-lysine at 0.1mg/ml (Invitrogen) for 2 hours at 37 degrees. Neurons were cultured in Neurobasal medium supplemented with 2% B-27, 0.5 mM L-glutamine, 10% penicillin/streptomycin, and half of the medium is replaced bi-weekly from day-in-vitro (DIV) 5 onwards. Experiments were conducted from DIV 20-23. All animal procedures were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) regulations.

Neuronal Stimulations and Silencing. We used a combination of 4-Aminopyridine (4AP), Bicuculline (Bic), and Forskolin at final concentrations of 100μM, 50μM, and 50μM respectively (4BF) to stimulate synaptic NMDA receptors and network activity and chemically induce LTP. The drugs were added to the culture medium for two to four hours. Activity silencing was done by adding tetrodotoxin (TTX) at a concentration of 1μM to the culture medium before treating the culture with the stimulation drugs.

Conventional Immunofluorescence. Cells were fixed with a solution containing 4% paraformaldehyde (PFA), 4% sucrose, and PBS for 15 minutes at room temperature (RT), blocked with a solution containing 10% goat serum, 2% bovine serum albumin (BSA), and PBS for 1 hour at RT. Rabbit-anti-NeuN (Thermo Fisher Scientific Cat# 711054, RRID:AB_2610583), mouse-anti-Arc (C7) (Santa Cruz Biotechnology Cat# sc-17839, RRID: AB_626696) and rabbit-anti-c-Fos (Santa Cruz Biotechnology Cat# sc-52, RRID:AB_2106783) primary antibody were used at 1:300. Cells were fixed with a solution containing 4% paraformaldehyde (PFA), 4% sucrose, and PBS for 1 hour at RT in a dilution buffer containing 1:1 block solution and PBS-Triton X solution at 1:300. Dishes were washed 3 times with PBS-Triton X and incubated with Alexa-Fluor 488 conjugated secondary antibody 1:1000 (Molecular Probes-Invitrogen) in dilution buffer for 1 hour at RT. Washing was repeated as per the above. The cells were subsequently incubated with 5μM DAPI for 5 minutes before being imaged.

Transduction. For calcium imaging, the GCaMP6m construct (pAAV.Syn.GCaMP6m.WPRE.SV40) was a gift from Douglas Kim & GENIE Project (Addgene viral prep # 100841-AAV9); http://n2t.net/addgene:100841; RRID:Addgene_100841), and transferred from University of Pennsylvania, Vector Core. It was transduced into the cells at a MOI of 1 x 10^5 on DIV9 to prevent virus toxicity.

Widefield Imaging. Fluorescence images were obtained using a motorized inverted wide-field epifluorescence microscope (Nikon Eclipse Ti-E), using Nikon 10X Plan Apo objective (N.A. = 0.4). Motorized excitation and emission filter wheels (Ladl electronics, Hawthorne, NY) fitted with a DAPI/CFP/YFP/DsRed quad filter set (#86010, Chroma, Rockingham, VT) were used together with filter cubes for DAPI, YFP and TxRed (Chroma) to select specific fluorescence signals.
Calcium imaging. Calcium imaging using widefield microscopy was done on neuronal cultures transduced with GCaMP6. Movies of 1-minute duration were taken at a frame rate of 5Hz at baseline, after 4BF treatment and post 4BF removal. Upon completion of calcium imaging, the culture was fixed and stained as described. Fig. 1A shows the calcium imaging and analysis pipeline.

Data analysis and graphs. Graphs were plotted using either GraphPad Prism Software (La Jolla, CA), or with MATLAB (ver. R2017b). Data are presented as mean with SEM. All custom-written codes for MATLAB used for the following analyses are available on GitHub at https://github.com/yuhengj/network_arc_dynamics.

Threshold selection. The determination of threshold for selection of positive populations was done systematically with custom-written MATLAB codes, which model the expression values as two separate normal distributions (an example of this is shown in Fig. 1E). As there is some overlap of the negative and positive populations, we aimed to reduce false positives by setting the threshold for positive cells to be at values higher than 99.7% (three times standard deviation) of the negative population.

Calcium transient and spike detection. Images were acquired with the Nikon NIS Elements software and subsequently processed with MATLAB, with the BioFormats plugin. Calcium transients from the whole network were computed based on the total fluorescence change over time. Photobleaching was accounted for with fluorescence intensity from cells that were silent during the recording (lowest tenth percentile of the whole population of cells). Peaks in fluorescence were detected using MATLAB function findpeaks. To obtain calcium signals from only the neuronal population, regions of interest (ROIs) were first determined from the DAPI images, and the expression levels of NeuN within the ROIs were quantified (Fig. 1B). NeuN positive threshold was determined by mixed population modelling with expression levels from all cells (Fig. 1E), and the threshold for negative population was selected to be 3 standard deviations from the population mean. These NeuN positive ROIs are then used to extract fluorescence signals from the calcium movies recorded. All pixels within each ROI are averaged to give a single time course, and calcium transients (ΔF/F) were calculated by subtracting each value with the mean of signals from the lowest 10% of neurons at each time point. This removes background and corrects for photobleaching over time. Spike times were then inferred from ΔF/F using a maximum a posteriori (MAP) estimate of the spike train developed by Joshua Vogelstein (Vogelstein 2010) named fast-oopsi. This implementation was adapted to be used in MATLAB as part of the FluoroSNNAP package developed by Patel and colleagues (Patel 2015), which is available for download at https://www.seas.upenn.edu/~molneuro/software.html. Firing rates of neurons were calculated as the number of spikes divided by the total recording time.

Neuronal activity correlations. Neuronal correlations were calculated using the spike time tiling coefficient (STTC) that was previously described (Cutts 2014). This was chosen as this measure is known to be independent of firing rate. Specifically, the correlation index (CI) is calculated according to equation (1) below:

\[
STTC = 1/2*((P_A T_A)/(1-P_A T_A) + (P_B T_B)/(1-P_B T_B))
\] (1)

where T_A is the proportion of total recording time which lies within plus minus delta t of any spike from spike times of A. P_A is the proportion of spikes from A which lie within a small time window (±Δt) of any spike from B. Δt was set to 50 ms according to the previous study (Cutts 2014). T_B and P_B are calculated correspondingly, for any two given spike trains A and B. A maximum value of +1 can be obtained for complete correlation. This pair-wise synchronization index for each neuron can be used to populate a adjacency matrix M of size n × n, where n = number of neurons in the network. We used
functions from the Brain Connectivity Toolbox (available for download at https://sites.google.com/site/bctnet) to edit the adjacency matrix.

Change in neuronal correlations. To determine the change in neuronal correlations of the whole network, we borrowed a measure developed for functional MRI that can represent all the connection strengths in the network, termed the Intrinsic Connectivity Distribution (ICD) (Scheinost 2012). This metric allows all of the neuronal correlations in the network to be captured with only a few parameters and eliminates the need for setting arbitrary thresholds. ICD models the distribution of all the correlations within the network first as a degree metric, from which a survival curve can be obtained and modelled as a stretched exponential with variance parameter ($\alpha$) and shape parameter ($\beta$) according to equation (2):

$$d(\tau, x, \alpha(x), \beta(x)) = e^{-\alpha(x)\beta(x)}$$  (2).

These parameters allow for a comparison of the distribution of correlations within a network, without the need for setting any arbitrary threshold for correlation and enables comparison of a network across different timepoints.

Firing rate analysis. The firing rates of individual neurons were calculated from NeuN positive DAPI ROIs and separated into Arc positive and negative values. The average firing rate at baseline was obtained for each culture by summing firing rate of individual neurons and dividing by the number of Arc-positive or Arc-negative neurons. The normalised change in firing rate during and after 4BF treatment was calculated by taking the change in firing as compared to baseline levels for each individual neuron and normalising it to the firing rate at baseline also for each individual neuron. The average value for each network (total of $n = 17$) was then computed by taking the mean.

Linear regression model for Arc positive percentage. A stepwise linear regression model was used to fit the Arc positive percentages data. The stepwiselm function from MATLAB was used to find optimal terms from a list of firing rates (initial, final and change), correlation index (CI) (initial, final and change), refinement status (binary) to explain the percentage of Arc. Analysis of variance of the linear model obtained was also conducted in MATLAB.

Increases and decreases in correlation index: Measures of STTC changes were used to determine changes in correlation index between different pairs of neurons after 4BF treatment. Correlations that initially had low correlation index (CI) which later became high are called “positive-change correlations”, whereas correlations initially having high CI but later having low CI were referred to as “negative-change correlations”. Specifically, positive-change correlations were defined as initial CI between the values of 0 and 0.5 and final CI between 0.5 and 1 while negative-change correlations are defined as initial CI between 0 and 0.5 and have final CI values between 0.5 and 1. The corresponding value of the percentage of Arc-positive to Arc-negative correlations is determined by counting the numbers of Arc-Arc correlations over the total number of correlations at each correlation threshold. For positive-change correlations, the threshold is based on the final CI while for negative-change correlations the thresholds are determined based on the initial CI.

Distance measure. Each neuronal nuclear ROI from which the immunofluorescent signal and calcium signal is extracted from, has a central coordinate (x and y coordinates based on pixels). Each type of connection between neuronal pairs therefore corresponds to a physical Euclidean distance that can be calculated based on the central coordinate. Each image frame was 1007 × 1007 pixels, with each pixel corresponding to 0.8 μm, giving a total area of 0.64 mm².

RESULTS
To achieve LTP in cultured hippocampal neurons, we made use of a combination of 4-aminopyridine, a blocker of presynaptic K,1 family K+ channels, bicuculline, a γ-aminobutyric acid (GABA) receptor antagonist, together with forskolin, an adenylyl cyclase activator which enhances NMDA-dependent LTP (Otmakhov 2004). This cocktail, subsequently referred to as 4BF, increased synchronised network bursting in hippocampal cultures. Calcium imaging and analysis were conducted as described in the Materials and Methods section and outlined in Fig. 1A.

4BF increases neuronal firing rate but decreases overall neuronal activity correlations

To assess the effects of 4BF on individual neurons, calcium traces for single neurons were extracted from the recordings. A representative raster plot of neuronal firing activity at baseline, during 4BF treatment and after 4BF treatment can be seen in Fig. 2A. We found that there was a significant increase in the firing rate in neurons following 4BF treatment (Fig. 2A & B). To determine the index of correlated activity between different neurons in the culture, the spike time tiling coefficient (STTC) was used, and the correlation index (CI) between all neuronal pairs were obtained (Cutts 2014). This revealed differences in initial culture states, with approximately 30% of the cultures (n = 8 out of total 28) having an overall high CI prior to the treatment by 4BF and the remaining dishes being low in neuronal correlation initially. Representative images of the correlations between neurons are represented as heatmaps in Fig. 3A & B, with an overall high CI initial state (Fig. 3B, first panel) or a low CI initial state (Fig. 3A, first panel). The average CI for each network was found to be decreased as compared to baseline (Fig. 2C), demonstrating an overall decrease in neuronal activity correlations after 4BF.

Refinement of network neuronal correlations by 4BF

To further investigate the distribution of activity correlations in the neuronal cultures, we examined the CI of each neuronal pair from the adjacency matrix and found that although there was a general reduction in CI after 4BF (Fig. 2C), some cultures had subsets of neurons that had a high CI after 4BF. This phenomenon was termed as refinement. Refinement happened independent of initial states of the network, in both cultures with high initial CI as well as networks with low initial CI (Fig. 3A & B). This refinement effect can also be visualised in the histogram of total flow (sum of all CIs through each node). As seen in Fig. 3C, there is a small peak at the region of high CI (marked out with the red box). Refinement after 4BF was observed in 60% of the cultures (n = 10 out of a total 17).

To better quantify this refinement in network neuronal correlations, it is of interest to specifically identify the neurons exhibiting high CI. However, inter-culture comparisons may not be ideal, as the threshold for CI can vary among cultures. Using a standardised threshold for all cultures to determine the refinement effect is therefore not ideal. To remedy this problem, we employed a previously developed measure named ICD for Intrinsic Connectivity Distribution (Scheinost 2012), which eliminates the need for setting arbitrary thresholds, and allows all of the correlation information to be captured with only a few parameters. Fig. 3C shows the degree distribution of CIs as histograms, before, during and after treatment with 4BF. Each degree distribution can give rise to a survival curve (Fig. 4A) by thresholding the degree distribution at different values (x-axis values). The values above the threshold degree were then integrated to obtain the normalised degree (y-axis values). This data was modelled as a stretched exponential according to equation (2). The variance (α) and parameter (β) can be obtained from the estimated function (Fig. 4A). These parameters allow for a quantification of the CI distribution within each network, before and after 4BF treatment (Fig. 4B). An ICD difference plot can be obtained by subtraction of the before and after plots and reveals changes in CI distribution (Fig. 4B). This was then utilised to effectively compare the correlations within the network before and after 4BF treatment. The ICD difference curves for all the cultures examined can be seen in Fig. 4C. Quantification of these curves was subsequently performed based on the width of half-peak height, the height and location of the peaks (Fig. 4D). For instance, a peak in ICD difference plot that is at a larger value of threshold indicates that there is...
a big change in CI before and after 4BF, and the width of half-peak height in the ICD difference plots indicates how widespread the change in CI is. For refinement, there is not only a global reduction in CI (corresponding to a larger threshold value at the peak) but also a wider distribution for the difference in CI (larger width of the ICD difference plots). The width measure and the peak location of the ICD difference plots were found to correlate well to whether the network has undergone refinement (Fig. 4E). The area under the ICD difference curves for all cultures were also obtained as a separate measure and it was found that larger area under the curve of ICD difference tended to correlate with effect of refinement (Fig. 4F). However, this measure failed to capture two (out of twenty-one) of the refined networks. The effect of refinement was verified visually using the adjacency matrices.

**Arc expression correlates with the final network CI and refinement**

To determine how refinement is related to Arc expression, the percentage of Arc positive neurons was determined by immunofluorescent staining and categorically compared as in Fig 5. Our results show that cultures expressing high levels of Arc are more likely to have undergone refinement. To test this observation more robustly, a linear regression model was used to fit the data, with the final Arc expression percentage as the outcome. Of all the input variables (including firing rate before and after 4BF, change in firing rate, connectivity indices before and after 4BF, change in connectivity index, and whether the network has undergone refinement), it was found that the final average network CI after 4BF treatment and refinement status can best predict the level of Arc expression in the network (Fig. 5B, Table 1). A total of n = 17 cultures was used for this analysis, with staining for both NeuN and Arc.

**Linear regression model**

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**Analysis of variance**

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$R^2_{\text{adjusted}} = 0.627$

**Table 1. Linear regression model variables and statistics.** Stepwise linear regression was used to determine variables that best explain the Arc-positive percentage (level of Arc expression in each culture). Final correlation index (average STTC) and the refinement status of the culture was found to be the best two variables. SE = standard error; T = t-statistics; P = p-values. Analysis of variance was done for this model with $F(2,14) = 14.46$. DF = degree of freedom; SS = sum squares; MS = mean squares; F = F-statistics; P = p-values. The squared multiple correlation $R^2 = 0.627$, indicating that 62.7% of the variability in the "Arc positive percentages" can explained by changes in "Final CI" and "Refinement status" of the cultures.

Arc is commonly used as a marker of neuronal activity, to determine if Arc-positive neurons have higher activity than Arc-negative neurons, we specifically examined the firing rates within Arc-positive neurons. Initial analysis of Arc-positive neurons within each network (from a total of n = 17 cultures) revealed that...
there was no difference in firing rates between Arc-positive (both NeuN and Arc positive) and Arc-negative neurons (NeuN labelled but Arc negative) (Fig. 1B & C). However, by examining the firing rate changes in individual neurons, we observe a slight increase in firing rate in Arc-positive neurons after 4BF treatment (Fig. 1F).

Correlated activity between Arc positive neurons is selectively increased and refined

To determine if there is any relationship between expression of Arc and neuronal activity correlations, we specifically examined the correlation indices (CI) of Arc positive neurons in each culture and those that do not express Arc (only labelled with the neuronal marker NeuN). Results indicate that Arc positive neurons in each culture exhibit higher connectivity than other non-Arc neurons (Fig. 7A, B). This is the case at baseline, during and also after 4BF treatment (Fig. 7A). Cultures tend to have an overall reduction in CI after 4BF treatment (Fig. 7C). By separating the analysis into Arc and non-Arc neurons, the Arc-positive neuronal population showed increased CIs after 4BF (Fig. 7B). Specifically, some cultures which exhibited lower initial CI increased in CI only in the Arc-positive population but not in the Arc negative population (Fig. 7B, C). This selective increase can also be seen from the adjacency matrix, a representative one being shown in Fig. 7D.

To investigate the effects of 4BF on neuronal activity correlations especially in the Arc positive neuronal population, all individual correlations between neuronal pairs were examined. These correlations can be grouped into correlations within the Arc-positive populations (that is the correlations between two Arc-positive neurons), correlations between Arc-positive neurons and Arc-negative neurons, and finally correlations between the Arc-negative neurons. The CI of these groups can be represented with a histogram as shown in Fig. 8A. It can be seen that the CI amongst Arc-positive neurons are higher than the CI between Arc and non-Arc and within non-Arc neurons at baseline. This means that neurons that initially had high CI are more likely to express Arc after the 4BF treatment. Subsequently, after 4BF treatment, the overall CI for all groups were reduced, but there remained a small number of correlations with high CI, especially from the Arc-Arc group (Fig. 8A, right panel insert). To examine the change for each correlation after 4BF treatment, the initial CI at baseline was plotted against the final CI after 4BF (Fig. 8B). This analysis revealed that many correlations have decreased CI (“negative-change correlations”) after 4BF treatment (Fig. 8B, negative-change correlations box, right panel insert). However, a subpopulation of correlations showed increase in CI (“positive-change correlations”) after 4BF treatment (Fig. 8B, positive-change correlations box, left panel insert). To determine the proportion of Arc-Arc correlations among the positive-change versus negative-change correlations, the percentages of Arc-Arc correlations were calculated as a function of the final (for positive-change) or initial (for negative-change) CI (labelled here as the correlation threshold), as seen in Fig. 8C. This reveals that there is a large proportion of Arc-Arc correlations with high CI after the 4BF treatment, and these correlations were initially low. This therefore supports the hypothesis that a subset of correlations between Arc-positive neurons are selectively increased after 4BF treatment.

Arc positive neurons are located physically closer to each other

To examine if there is any relationship between the status of Arc expression and the physical distances between pairs of neurons, the coordinates of the neurons in each culture were obtained. Distances between each pair of neurons were calculated as described in the Materials and Methods section. Fig. 9A shows the values of distances between pairs of neurons according to their Arc expression status. We found that the distances between Arc-positive neurons are generally shorter than that between Arc and non-Arc and also between the non-Arc neuron pairs. This means that Arc-positive neurons are likely to be closely located in the vicinity of each other. To determine if there is any correlation between the CI and physical distance between each pair of neurons, we examined the association between distances and
CI of each neuronal pair both at baseline (Fig. 9B, left panel) and after 4BF (Fig. 9B, right panel). 4BF does not significantly change the distribution of distance versus CI of the neuronal pairs. Most of the distances are short-ranged, with a few long-ranged ones that extend up to 2.4 mm. These long-range distances tend to be associated with CI of low values around zero. This is the case both at baseline and also after 4BF treatment. The zoomed-in insert on the right panel of Fig. 9B shows that correlations with high CI after 4BF treatment are likely to have short-range distances between pairs of neurons of which at least one is Arc-positive. In Fig. 9C high CI between Arc and non-Arc neurons marked out with arrows. This distance analysis shows that these correlations that are marked out likely represent neuronal pairs that are in close physical proximity to each other. To determine if there is any difference in physical distance between positive-change versus negative-change correlations, a similar analysis was done as in the previous section. There is a general down sloping trend for both types of connections, showing that larger changes in CI (both positive and negative) tend to be short ranged (Fig. 9D).

**Arc positive cells are mostly a subset of c-Fos cells.**

To investigate the effects of 4BF treatment on the expression of both Arc and c-Fos in vitro, the same drug treatment protocol was used and the expression levels of both these proteins were assessed simultaneously using immunofluorescence staining. By co-labelling both Arc and c-Fos, it was found that most of the Arc expressing cells were also c-Fos-positive. This was done in a total of n = 11 cultures. Thresholds for detection of positive expression were computed as described in the methods, and the numbers of Arc and/or c-Fos positive neurons were quantified and their proportions determined across these eleven cultures. The majority of neurons were positive for both Arc and c-Fos, with some that were only positive for c-Fos and only very few cells that were only positive for Arc (Fig. 10A). The pie chart in Fig. 10B shows that an average of 16.2% of all cells in the culture express Arc and/or c-Fos after 4BF treatment. Among the Arc-positive neurons, 82.7% of these are also c-Fos-positive (Fig. 10B).

**Arc and c-Fos expression as a combinatorial code for correlation strength pruning.**

Based on the expression profile of Arc and c-Fos, the cells were grouped into Arc+c-Fos+, Arc+c-Fos−, Arc−c-Fos+, and Arc−c-Fos−. The intra group and across group correlations in activity between pairs of neurons can thus be classified into ten different sets. To examine the change across time after 4BF treatment on each set of correlations, probability density distributions of each set of correlations were plotted using a non-kernel density estimation method (Fig. 11A). The intra-group correlation sets (Fig. 11A, subplots I, V, VIII, X) were also plotted on the same graphs in Fig. 11B to highlight the differences across different intra-group sets. Results show that double positive neuronal correlations were the strongest prior to 4BF treatment (Fig. 11B, left panel). The correlations between neurons expressing only Arc but not c-Fos to other neurons were specifically down-regulated (Fig. 11A, subplots II, V, VII, Fig. 11B). Interestingly, the c-Fos-only correlations were not changed much after 4BF (Fig. 10A, subplots III, VI, VIII, IX). These results support the hypothesis that there is a combinatorial code by the expression of Arc and/or c-Fos in association with changes in correlated activity between these neurons.
DISCUSSION

We have made use of pharmacological treatment by 4BF on hippocampal neuronal cells, which have been established in previous studies to induce LTP, to show that there is a global reduction in network neuronal activity correlations while a subset of correlations between some of the neurons showed a selective increase. This refinement effect correlates specifically with Arc expression in the network, with Arc-positive neurons demonstrating increase correlated activity while non-Arc neurons demonstrating a decrease in correlated activity. The expression patterns of IEGs Arc and c-Fos were shown to be mostly overlapping, but Arc is more selectively expressed than c-Fos, after induction of chemical LTP by 4BF. The expression status of Arc and c-Fos in the neurons is associated with distinct variations in correlation strength changes before and after 4BF treatment.

Correlated activity and functional-connectivity

The overall goal of this study was to correlate expression of the two IEGs Arc and c-Fos in individual neurons with changes in activity and functional-connectivity induced by chemical LTP. Changes in correlated activity between a pair of neurons is indicative of alterations in functional-connectivity. However, it does not imply that the synaptic connections between these two neurons have been altered. In fact, it is possible that the two neurons are not synaptically connected, but both receive inputs from the same subset of neurons and their functional connectivity with this subset was altered in the same direction. Given the propensity of these networks of cultured neurons for highly synchronized activity (Drania 2013), this is not an unlikely scenario. Whereas no firm conclusions can be made regarding changes in functional-connectivity for specific neuron pairs, our finding that the subset of Arc-positive neurons displayed a selective increase in activity correlation, suggests that functional-connectivity was strengthened between the neurons of this subset.

Association between IEG expression and correlations in activity

This study aims to relate neuronal activity-dependent expression of the IEGs Arc and c-Fos on the level of individual cells to changes in neuronal activity correlations in the network. We have provided an interesting perspective of the relationship between correlated neuronal activity in a population of cells and the expression of two IEGs, Arc and c-Fos, on the individual cellular level. However, this work is mostly correlative as we have not determined the mechanism underlying the changes in correlated firing in the culture. We have made some attempts to address this by studying the effects of Arc knock-down on the correlated activities of the neuronal cultures after 4BF. This was done using a previously validated short hairpin RNA (shRNA) construct targeting the coding region of Arc, which has been shown to be able to suppress Arc induction following 4BF treatment (Leung et al., 2019). However, the introduction of two viral vectors to the neurons at high MOI resulted in significant cell death. We therefore did not include these networks for further analysis. We believe that the effects of IEG manipulation on functional connectivity changes is an important next step to further probe the causative relationships that we have observed. Another limitation of our study is that LTP was not measured directly as a result of 4BF. Although 4BF has been previously used to induce LTP, the establishment of LTP in the neuronal network was not empirically determined in our cultures. Our results showed that 4BF resulted in a global reduction of correlated neuronal activity, which seems counterintuitive to effects of LTP. However, we propose that the observed refinement effect may be a network level response in aiding LTP of specific connections, as more contrast is provided to the connections which are strengthened. This effect of network refinement is discussed in more details in a subsequent section.

Potential sources of error and caveats

In our study, we have identified neurons by NeuN labelling, but have not distinguished excitatory projection neurons and inhibitory interneurons. Therefore, differences in the GABAergic inter-neuronal...
Network refinement as an emergent network property after chemical LTP

The potentiation effects of LTP have been well-studied on the cellular level, with the measurable end result usually being increases in excitatory postsynaptic potentials (EPSPs) or changes in spine size. However, few studies have investigated the effects of changes in neuronal firing behaviour (Marder 2004). One such study by Chiappalone et al. (Chiappalone 2008) using neuronal cultures grown on MEAs found that a tetanus stimulus can induce an overall decrease in evoked network activity, although the same stimulus has been previously shown to induce potentiation of specific pathways (Jimbo 1999). This is consistent with the effects of refinement in correlated activity after 4BF treatment. It has long been thought that plasticity mechanisms cause networks to destabilise (Abbott 2000), and many cellular and network mechanisms, generally termed as homeostatic plasticity, have been discovered to push their state back to baseline (Turrigiano 2012). Among the few cultures that had a decrease in firing rate in response to 4BF, all of them had high network average CI. However, there is a dissociation between the changes in firing rate and correlated activity following 4BF treatment. Although most of the cultures showed an increase in firing rate, there is a decrease in network average CI in all the cultures. Homeostatic synaptic scaling that is required for firing rate modifications in response to prolonged neuronal activity is thought to involve the differential expression of AMPA receptor subunits (Goold 2010) and a reduction of AMPA receptors at the synapse (Seeburg 2008). It is known that Arc over-expression in hippocampal cultures blocks homeostatic upregulation of AMPA receptors in chronic inactivity (Shepherd 2006). The synaptic scaling that results from chronic activity is commonly observed at 48 hours after stimulus onset (Turrigiano 1998). The changes reported here on network neuronal correlations take place in a shorter time frame, around 6 to 8 hours after 4BF is applied, and therefore the changes in correlated activity may not be a result of the usual mechanisms known in synaptic scaling.

It is known that when LTP reaches saturation, no further learning can occur (Moser 1998). Thus, down-regulation of correlated activity in response to prolonged activity is necessary for the network to continue coding useful information. Besides cell autonomous mechanisms of achieving homeostasis, networks are known to maintain stability through coordinated inter-cellular interactions (Maffei 2009). One feature worthy of mentioning is the criticality of neuronal networks. Criticality is a network state that maximises information processing capacities by having high level of responsiveness to external stimuli (Rocha 2018). Using neural networks in silico, it has been demonstrated previously that plasticity mechanisms can tune a network to criticality (Rubinov 2011). Effects of 4BF could push the network to criticality and possibly to a supercritical state, where the threshold of activation is high and thus reflects a global reduction in
correlated activity. Pharmacological induction, such as the 4BF treatment used here, is often thought to
be a strong inducing stimulus that may not be physiologically relevant. However, repeated activation of
NMDA receptors is known to be important for memory consolidation (Wittenberg 2002). It has also
been shown that during sleep, the majority of cortical synapses are up-regulated, supporting the
hypothesis that slow oscillations observed during sleep potentiate synapses that were depressed due to
persistent activities during the day (Timofeev 2001). The refinement process could act to increase the
contrast of significant correlations in the network by modulating network threshold. Refinement of
network neuronal correlations that we have shown here in this study is an emergent network property in
response to chemical LTP.

**Arc-positive neuronal connections exhibit increased activity correlation changes**

IEG expression is often used as a marker of neuronal activity. This is consistent with our findings of
increased firing rate changes in Arc-positive neurons. However, this effect on increased firing rate is not
uniform across all Arc-positive neurons in a given network. This can be seen from the lack of difference
between firing rate changes of Arc-positive and Arc-negative neurons when the analysis was conducted
on a per culture level. This means that within each network, the population of Arc positive neurons have
variable changes in firing rate, and not all Arc-positive neurons demonstrate increased neuronal activity.

We showed that in networks where the effect of 4BF on network refinement is largest, there is
correspondingly a high level of Arc expression. Subsequently, not all the neurons in the network express
Arc, even though they might participate in the increased network firing. In particular, Arc expression is
high in neurons with strongly modulated correlated activity, and especially for positive-change
correlations. This is consistent with findings in *vitro* by Mahringer *et al.* (Mahringer 2019) where they found
that neurons undergoing the strongest learning-related changes in activity would exhibit increased levels
of IEG expression. Furthermore, subsequent analysis with c-Fos also demonstrated that Arc+cFos−
nurons tend to have negative-change correlations. This contributes to the idea that Arc-positive neurons
have increased correlation changes (either increased or decreased) after chemical LTP. Arc may be poised
to perform a role in this process, as it has been shown to play a part in both LTP as well as long-term
depression (LTD) (Bramham 2008). Increased functional-connectivity changes of Arc-positive neurons
supports the idea that Arc expression is not primarily a marker of neural activity. The current theory of
engram formation assumes that all cells that are more active during encoding go on to express the IEG,
subsequently become more connected to each other and form the memory engram. Our results suggest
that not all Arc-expressing neurons have an increased firing rate after the stimulus. Neurons that are
activated during the process of encoding thus do not undergo uniform increase in correlated activity. Our
results show that selective neuronal pairs particularly within the IEG-expressing population demonstrate
increased correlated activity, with other pairs showing decreased correlated activity. This refinement effect
is particularly apparent within the Arc expressing neuronal population.

**Expression of Arc may depend on network architecture**

We have shown that there is a negative correlation between physical distance of neuron pairs and their
functional connectivity. Correspondingly, connections that exhibit large activity correlation changes after
4BF treatment tend to have closer physical proximity and involve at least one Arc-positive neuron. This
relationship between correlated activity and anatomical distance is consistent with previous reports of
findings on the brain level. For instance, fMRI studies have been shown that the functional connectivity
is stronger between regions with shorter physical distance and conversely, correlated activity decreases as
the physical distance between brain regions increases (Salvador 2005, Bellec 2006). This pattern of
functional connectivity variation with physical distance aims to minimise wiring cost and thus follows
an economical principle in circuit organisation. Such a pattern of activity correlations may also be at play in
*vitro* for individual connections between neurons. 4BF acts to increase synchronised bursting of the
network, however, only a selective subset of neurons express IEGs such as Arc. We have shown here that these selected subsets tend to be located physically closer to each other. The expression of Arc, therefore, may depend on the architecture of the network. Pre-existing short-range connections could undergo selective increase in correlated activity.

**Arc and/or c-Fos expression correlates with distinct changes in neuronal correlations**

Our results showed that Arc and c-Fos are expressed in subsets of neurons after 4BF induction, and large correlated activity changes are likely to happen among Arc-positive neurons. Further analysis of the co-staining with c-Fos reveals that correlated activity within the Arc+cFos− subgroup of neurons are mostly decreased. On the other hand, there is a high degree of correlated activity among the double-positive (Arc+cFos+) neurons, the Arc=cFos+ neurons and between these two groups. Arc and c-Fos have been shown to act in combination to specify whether hippocampal neurons are involved in memory reactivation using a novel environment exposure protocol in mice (Jaeger 2018). In their experiments, both Arc and c-Fos are expressed abundantly 1-hour post novel environment exposure, and highly overlap, but only Arc persists through 5 hours after the exposure. As such, doubly positive Arc+cFos+ neurons are reactivated or newly activated upon re-exposure to the same context, whereas Arc+cFos− neurons are not reactivated upon exposure to a new context. Our results show that Arc expression in neurons is correlated with presence of strongly modulated changes in CI. The concurrent presence of c-Fos in these neurons correlate with the direction of the modulation of changes in CI. This is in line with the hypothesis that expression of Arc and c-Fos may act as a combinatorial code for distinguishing the subpopulations of neurons that undergo different types of activity-dependent plasticity, though confirmation of this hypothesis would require further experiments to demonstrate the causality involved in this relationship.

**Dynamic interplay between network activity, IEG expression and plasticity**

Conventionally, studies of neuronal activity and IEG expression are mostly based on the single cell level whereas network activity is recorded on the neuronal population level. Our work here correlates the expression of the two IEGs Arc and c-Fos in individual neurons with changes in activity and functional-connectivity induced by chemical LTP. Some others like Mahringer et al (Mahringer 2019) have examined the expression of IEGs in vivo and linked it to the activity of neurons during specific tasks, which has the benefit of relating individual neuronal responses to specific stimuli presented to the animal. We believe that our study has added to the idea that there is a dynamic interplay between IEG expression and neuronal activity in the network. To further probe the links between IEG expression and connectivity changes, specific stimulus patterns selectively activating subsets of neurons could be used, for instance using optogenetic methods. This would provide a direct assessment of the causal relationship between changes in network activity and molecular changes within individual cells, and thereby linking IEG expression, synaptic plasticity, and network connectivity.

**Summary**

We investigated the network changes that can occur after induction of LTP in vitro and found that chemical LTP induction by 4BF resulted in an emergent effect of refinement of the correlated neuronal activities. Arc expression correlates with effects of 4BF, particularly on network refinement, and Arc-positive neurons exhibit more changes in correlated activity after 4BF. We also found that the architecture of the network may affect Arc expression as Arc positive neurons are located closer to each other in the network. In terms of the expression of IEGs Arc and c-Fos, we found that Arc is more selectively expressed after 4BF. The differential expression of these two IEGs were associated with changes in correlated neuronal activity. With these results we have uncovered some interesting and important links between correlated neuronal activity, and IEG expression.
Figure 1. Calcium imaging pipeline and data analysis. A) Schematic of calcium imaging: cells transduced with GCaMP6 are imaged under widefield epifluorescence imaging at baseline and after 4BF treatment. Calcium movies are processed to extract the calcium imaging data of individual neurons, which are converted into spike times using methods described. The correlation between neuronal spike times is calculated using the spike time tiling coefficient (STTC). Cells were fixed and stained with the desired markers (NeuN with Arc or Arc with c-Fos). These were then used as identity markers for subsequent connectivity analysis. B) Representative immunofluorescence staining image showing NeuN and DAPI. Extraction of calcium waves of individual neurons are based on selection of regions of interest (ROIs) by segmentation of the DAPI signals. The intensity of NeuN staining was determined within each DAPI ROI. These expression values were modelled as two separate normal distributions (similar to panel E), and the threshold for selection of positive populations was determined based on the distributions as described in the Methods. C) Following thresholding, the NeuN-positive population from each network can be labelled as shown, based on ROIs from DAPI segmentation. D) The accuracy of thresholding can be verified visually by overlaying the NeuN-positive ROIs onto immunostaining images of NeuN. E) Threshold selection was done based on mixed population modelling. Values of expression intensities was plotted according to their probability distributions and modelled as two normal distributions. The mean and standard deviations of these two distributions were obtained computationally and threshold was selected based on these values as described in the methods section. This method was also used to determine the threshold for Arc-positive and c-Fos positive populations. F) Representative immunofluorescence staining image showing Arc and c-Fos with DAPI. G) Representative post analysis reconstruction image indicating ROI determination by DAPI and allocation of ROIs according to Arc and c-Fos identity as determined by their expression levels.

Figure 2. Neurons exhibit increased firing rate but decreased CI after 4BF treatment. A) Representative raster plots of a culture at baseline and 3 hours after being treated with 4BF. B) Quantification of firing rates during and after 4BF treatment, both are compared and normalised to firing rates for each culture at baseline. Normalised difference in firing rate during 4BF = -0.0373, SEM = 0.0326. Normalised difference in firing rate post 4BF = 0.185, SEM = 0.0639, n = 17 for both. C) Quantification of STTC during and after 4BF treatment, both are compared and normalised to the STTC for each culture at baseline. Normalised difference in STTC during 4BF = -0.127, SEM = 0.0569. Normalised difference in STTC post 4BF = 0.330, SEM = 0.0518, n = 17 for both. Firing rates were quantified from cultures that were subsequently stained with NeuN marker (n = 17 cultures), and only neuronal cells that are positive for NeuN were selectively used for quantification of firing rates.

Figure 3. Refinement of correlated activity after 4BF. A) A culture showing an initial state of low CI, with refinement after 4BF. B) A culture showing an initial state of high CI with refinement after 4BF. C) Histogram of total flow (sum of all CI through each node) at baseline, and during and post 4BF for a representative culture. There is a right shift in the distribution with a higher proportion of CI in the culture being of higher value post 4BF.

Figure 4. The ICD measure for describing neuronal correlations. A) Survival curve obtained by thresholding the degree distribution at different values (x-axis values). The values above the threshold degree were integrated to obtain the normalised degree (y-axis values). Data at different values shown in blue, modelling of stretched exponential with variance (\(\alpha\)) and parameter (\(\beta\)) (equation (2), methods), shown in red as the fitted curve. B) The ICD distribution at baseline (blue) and after 4BF treatment (orange), and the ICD difference plot (red dotted line) for a sample culture. C) ICD different plots for all cultures with each colour representing a different culture. The width, peak height and peak location of
these curves can be obtained. D) Different measures that can be obtained from the ICD difference plot: 
width of half-peak height, height and location of peaks. E) Cultures that have not undergone refinement 
have low peak widths and peak locations (marked in blue ‘o’), n = 7. Cultures that have undergone 
refinement have large peak widths and peak locations (marked in red ‘o’), n =10. Refinement was verified 
visually based on the connectivity matrices. F) The area under the ICD difference curves was also 
computed for each culture and the distribution of the areas is plotted. The effects of refinement correlate 
with larger area under the ICD difference curves. However, this measure failed to capture 2 of the refined 
cultures (out of a total of 10).

Figure 5. Arc expression and neuronal activity. A) Graph showing change in firing rate after 4BF vs 
percentage of Arc expression in the culture. Side panel pie charts: a reduction in firing rate is associated 
with a low level of Arc expression (14%) while an increase in firing rate is associated with a higher level of 
Arc expression (50%). This was found from a total of n =17 cultures. B) Linear regression model with 
stepwise addition of factors CI (STTC values), firing rate, state of refinement (binary factor). It was found 
that the final STTC value together with the state of refinement are most able to explain the level of Arc 
expression after 4BF.

Figure 6. Difference in firing rates between Arc-positive and Arc-negative neurons. A) Firing rate 
of Arc-positive and Arc-negative neurons in each culture. B & C) Change in firing rate of Arc-positive 
and Arc-negative neurons in each culture, normalised to baseline, during and after 4BF treatment (n=17 
cultures). D) Firing rate of all Arc-positive (blue) and Arc-negative (orange) neurons across all cultures 
tested (n=17 cultures). E & F) Change in firing rate of all Arc-positive (blue) and Arc-negative (orange) 
neurons during and after 4BF respectively.

Figure 7. Difference in correlated activity between Arc-positive and Arc-negative neurons. A) CI 
between Arc positive neurons in each culture and those that do not have Arc. Arc positive neurons in 
each culture exhibit higher CI than other non-Arc neurons at baseline, during and also after 4BF 
treatment. Mean CI between Arc-Arc neurons at baseline = 0.147, SEM = 0.0258. Mean CI between Arc-
NeuN neurons at baseline = 0.0954, SEM = 0.0197. Mean CI between NeuN-NeuN neurons at baseline 
= 0.0686, SEM = 0.0166. Mean CI between Arc-Arc neurons during 4BF = 0.0967, SEM = 0.0157. 
Mean CI between Arc-NeuN neurons during 4BF = 0.0562, SEM = 0.00928. Mean CI between NeuN-
NeuN neurons during 4BF = 0.0415, SEM = 0.00844. Mean CI between of Arc-Arc neurons after 4BF = 
0.0336, SEM = 0.0195. Mean CI between of Arc-NeuN neurons after 4BF = 0.00108, SEM = 0.00598. 
Mean CI between of NeuN-NeuN neurons after 4BF = -0.00127, SEM = 0.00474. A total of n = 17 
cultures were examined. B) Some of the Arc-positive neuronal population increased in CI after 4BF. 
These were cultures which exhibited lower initial CI. C) Such increase is not seen in the Arc-negative 
population. D) Representative adjacency matrices of a culture at baseline and after 4BF treatment, 
showing selective increase and refinement in the Arc-positive neurons.

Figure 8. Difference in CI between neuron Arc-positive neurons and those that do not. A) Pairs of 
neurons are grouped into: Arc and Arc (blue), Arc and non-Arc (orange), and non-Arc and non-Arc 
(yellow). The distribution of CI according to their groups (labelled in different colours) are shown in the 
histograms, with the left panel being the CI between all pairs of neurons at baseline and the right panel 
being the CI between all pairs of neurons after 4BF treatment. The x-axis represents the range of CI, 
which are mostly between the values of -0.2 to 1. The y-axis represents the frequency at which each CI 
interval occurs, with a peak at around zero. This indicates that most of the CI are of low values. The 
insert on the left panel is a zoomed-in view of the higher CI between 0.4 to 1. This shows that the highest 
CI among pairs of neurons after 4BF treatment occur between Arc-positive and Arc-positive neurons. B) 
Scatter plot of CI before 4BF (at baseline) and after 4BF treatment. Left dotted box indicates CI that are
of low values initially that have increased in value after 4BF treatment (positive-change correlations), whereas right dotted box marks out CI that are initially of high values that have decreased after 4BF treatment (negative-change correlations). The lower left and right panels are zoomed in views of positive-change and negative-change correlations respectively. C) The percentage of Arc-positive to Arc-positive pairs is determined by counting the numbers of Arc-Arc pairs over the total number of neuronal pairs in the culture at each correlation threshold. Positive-change correlations are those that have initial CI between the values of 0 and 0.5 and final values between 0.5 and 1 while negative-change correlations are defined as those that are initially between 0 and 0.5 and have final values between 0.5 and 1. The x-axis represents the range of correlation thresholds above which the percentage Arc-Arc pairs are evaluated; for positive-change correlations, the threshold is based on the final CI while for negative-change correlations the thresholds are determined based on the initial CI.

**Figure 9. Distance measures of different types of neuronal pairs.** A) Distances between pairs of neurons for each type of neuronal pair. Arc-Arc: Arc-positive neurons and Arc positive neurons. Arc-NeuN: Arc-positive neurons and Arc-negative (non-Arc) neurons. NeuN-NeuN: Arc-negative neurons and Arc-negative neurons. B) Distances between pairs of neurons vs CI at baseline (left panel) and after 4BF (right panel). Different types of neuronal pairs marked with separate types of markers (see legend). Zoomed-in insert on the right panel shows the same graph with CI between 0.8 and 1. C) Representative adjacency matrix after 4BF treatment (as in Figure 4.3D). Dotted boxes show the CI profile between Arc-positive and Arc-negative neurons, with arrows indicating high CI. D) Positive-change correlations are those that have initial CI between the values of 0 and 0.5 and final values between 0.5 and 1 while negative-change correlations are defined as those with CI that are initially between 0 and 0.5 and final values between 0.5 and 1. The x-axis represents the range of correlation thresholds above which the percentage Arc-Arc neuron pairs are evaluated; for positive-change correlations, the threshold is based on the final CI while for negative-change correlations the thresholds are determined based on the initial CI. The y-axis shows values of average distances of all neuronal pairs above the threshold value in x. Distance measured in pixels, with each pixel corresponding to 0.8 μm.

**Figure 10. Arc and c-Fos expression after 4BF.** A) Expression of Arc and c-Fos in individual neurons from a single frame. This shows the majority of the cells are both Arc and c-Fos positive, with a good number of c-Fos only expressing cells but few Arc only expressing neurons. B) Pie chart with Arc and/or c-Fos-positive percentages after 4BF treatment. C) Arc and c-Fos expression at baseline and after 4BF treatment.

**Figure 11. Probability density plots of CI distributions of different populations of neurons.** (x-axis represents the connection strengths and y-axis represents frequency of the strengths for all plots.) A) Probability density plots of CI distributions between different groups of neurons that express Arc and/or c-Fos or none of these markers. Given that there are four groups of cells depending on the expression status (as listed in the top row and similarly in the first column), there are ten different sets of connections between these four groups. The CI between these 10 sets are non-overlapping and are represented in the subplots I to X. The row and column headers are labelled with the identity of the groups of the neuronal pair. B) The intra-group pairs (subplots along the diagonal, I, V, VIII and X) are extracted and plotted using different colours on the same graph for baseline, during 4BF treatment and after 4BF treatment.


Wee, C. L., S. Teo, N. E. Oey, G. D. Wright, H. M. VanDongen and A. M. VanDongen (2014). "Nuclear Arc Interacts with the Histone Acetyltransferase Tip60 to Modify H4K12 Acetylation(1,2,3)." eNeuro 1(1).

