Temporal Pattern of Synaptic Activation Determines the Longevity of Structural Plasticity at Single Dendritic Spines

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Highlights

- Naturalistic stimulation of single spines induces long lasting structural plasticity that depends on the temporal distribution of the synaptic events.
- Structural plasticity induced by naturalistic stimulation patterns requires NMDA receptor activation and new protein-synthesis.
- Rapid spine structural dynamics during naturalistic activity, but not regular patterns, predict the longevity of subsequent structural plasticity.

Summary

Individual synapses are the points at which information is passed into neurons, yet it is unknown how the diverse patterns of activity that are observed *in vivo* effect plasticity at the level of single inputs. Here, we wanted to determine what are the structural plasticity consequences of naturalistic patterns of activity at single spines, as these reflect changes in synaptic efficacy. Utilizing two-photon fluorescence imaging and glutamate uncaging, we studied structural plasticity of individual CA1 hippocampal dendritic spines using activation patterns sampled from a Poisson distribution, which resemble endogenous firing patterns from their CA3 inputs. We found that while the majority of inputs initially undergo structural changes, the robustness of this plasticity is determined by the timing structure of the Poisson sampled naturalistic stimulation patterns. Further, we found that structural plasticity elicited by these naturalistic patterns is both NMDAR and protein synthesis dependent, consistent with requirements for other forms of plasticity. Lastly, we found that during the delivery of naturalistic activity patterns, spines underwent rapid and dynamic structural growth that predicted the longevity of plasticity, which was not the case during tonic stimulation protocols. These data suggest that dendritic spines are able to integrate incoming temporal information and accordingly modulate the longevity of plasticity that is induced.

Keywords

Single spine plasticity, glutamate uncaging, structural plasticity, structural changes, spine growth, naturalistic stimulation patterns, long term potentiation (LTP), long lasting plasticity, protein synthesis dependent plasticity, two-photon imaging.

Introduction

Information is believed to be encoded at the cellular level through activity dependent alterations in synaptic weights, which are correlated with structural modifications of dendritic spines (Matsuzaki et al., 2004; Malenka and Bear, 2004). These are the major sites where excitatory synapses are located and their morphology is highly regulated by incoming activity (Bartol et al., 2015; Holler et al., 2021). Through two-photon imaging and glutamate uncaging, neuronal structure and function can be probed with high temporal and spatial resolution at single synapses, allowing for the identification of a linear relationship between the current amplitude of a synapse and the volume of the spine on which it is located (Smith et al., 2003; Losonczy and Magee 2006). Further, light mediated release of regularly-spaced pulses of glutamate induce both synaptic potentiation and long lasting spine growth at single spines demonstrating that changes in spine size can be used as a proxy for functional plasticity (Matsuzaki et al., 2004; Harvey and Svoboda, 2007; Govindarajan et al., 2011). Such structural plasticity can either be short- or long-lasting, depending on the availability of plasticity related proteins (Govindarajan et al., 2011), similar to the case during functional plasticity (Frey and Morris 1997; McGaugh 2000; Fonseca et al., 2006).

Much of our understanding of synaptic plasticity rules has been achieved through the delivery of regularly-spaced temporal patterns of activity, such as high or low frequency stimulation trains (Bliss and Lomo, 1973; Ito and Kano, 1982; Bear and Malenka, 1994). However, endogenous activity in the brain is organized differently, and firing patterns are more accurately described by Poisson-like distributions, in which spiking events occur independently over time with intervals spanning an exponential range (Softky and Koch, 1993; Dobrunz and Stevens, 1999; Frerking et al., 2005; Chokshi et al. 2021). Indeed, the response properties of single neurons and local circuits are fundamentally different for regularly

spaced stimuli compared to naturalistic or noisy stimuli (Mainen and Sejnowski, 1995; Vinje et al., 2002; Faisal et al., 2008; Herikstad et al., 2011).

Therefore, we aimed to determine what are the structural plasticity consequences of naturalistic based patterns of activity at single spines. We developed optical stimulation paradigms with Poisson timing structures to mimic the irregularity of endogenous activity in the hippocampus. We used two-photon glutamate uncaging and imaging to deliver these patterns to single dendritic spines in CA1 pyramidal neurons and tracked the resulting structural plasticity over different time scales. We find that the temporal pattern of activation determines the longevity of plasticity, indicating that single dendritic spines integrate transient information into either short or long-lasting structural plasticity in an activity dependent manner. The critical variable which gave rise to this difference in plasticity was in the spacing of the patterns: when stimuli were spread evenly across the epoch they induced more robust plasticity, while those with stimuli massed towards either the beginning or the end of the patterns were less efficient at inducing sustained structural plasticity. We also observed that across the population of potentiated spines, the degree of structural growth during these stimulations varied and was highly predictive of the longevity of structural plasticity that spines would express. Finally, we determined that the induction of structural plasticity by these patterns requires activity through NMDA receptors, as well as new protein synthesis, confirming the involvement of known synaptic plasticity mechanisms for the expression of long lasting structural modifications. Thus, we demonstrate for the first time that naturalistic based stimulation patterns can elicit plasticity at single inputs, and that the robustness of the resulting plasticity is dependent on the temporal structure of the activity.

Results

Temporal Structure of Naturalistic Stimulation Patterns Determines the Longevity of Plasticity

A widely used paradigm for the induction of long-term potentiation (LTP) at single spines involves light mediated delivery of 30 pulses of glutamate (4ms-long pulse-width, at 0.5 Hz, lasting 60 seconds) (Harvey and Svoboda, 2007; Harvey et al. 2008; Govindarajan et al., 2011; Hill and Zito, 2013; Bosch et al., 2014; Hobbiss et al., 2018), which we will refer to as 30-Reg (Reg: Regularity of spacing). We wanted to establish a naturalistic based uncaging stimulation pattern that we could compare to the known structural and functional outcomes of the 30-Reg stimulation. We chose paradigms with timing structures that follow a Poisson distribution, as the irregularly structured nature of endogenous activity in the hippocampus is well modelled by this distribution (Rich et al., 2014). It is also consistent with the range of firing schemes observed in behaving animals in CA3 pyramidal neuron populations, which input onto CA1 dendrites (Frerking et al., 2005). We sampled multiple temporal patterns from a homogeneous Poisson process (HPP) with an expected number of events equal to 30, to approximate our standard paradigm (λ=30, Figure 1A, see Methods for details). Among this distribution, we selected only those patterns containing exactly 30 pulses, to match our established plasticity protocol, as well as to control that the same total amount of glutamate would be delivered over the stimulation window across paradigms. Due to the probabilistic nature of the Poisson sampled patterns, only 740 patterns out of the 10000 generated had exactly 30 pulses in 60s (Figure 1A inset). We sorted the patterns according to how events were distributed over three equal time bins: either a pattern contained a pseudo-uniform distribution (with 10 pulses in each 20s bin), or pulses were skewed towards occurring predominantly at the beginning or end of the stimulation period (during the first or last 20s bin). We refer to these patterns as naturalistic stimulation patterns

(NSP), and we selected one representative from each group: NSP-Uniform (NSP-Uni), NSP-Beginning (NSP-Beg) and NSP-End, respectively (Figure 1B and Supplementary Figure 1). Thus, we had one homogeneously distributed Poisson pattern (NSP-Uni) and two non-homogeneously distributed Poisson patterns (NSP-Beg and NSP-End) that emerged from the same distribution, which we next used to evaluate structural plasticity at single spines.

Using each of these defined patterns (NSP-Uni, NSP-Beg, NSP-End) as well as the 30-Reg LTP paradigm (Figure 1B), we utilized two-photon mediated glutamate uncaging and imaging to induce plasticity at individual spines (Figure 1B-below and 1C) and quantified the resulting structural changes before, during and after glutamate delivery (Figure 2, Supplementary Figure 6). We first tested whether the homogeneously distributed Poisson pattern (NSP-Uni) was capable of inducing structural plasticity at single inputs. We found that indeed, stimulation with the NSP-Uni pattern led to long-lasting growth of individual spines ($\Delta V_{NSP-Uni}$=154±13%, Mean±SEM, p=6.9471^{-4}, averaged over the last 45 min, stimulated vs unstimulated neighbors, Mann-Whitney U) (Figure 2C-D and Supplementary Figure 2). The amount of growth observed with the NSP-Uni stimulation was similar to that induced with the 30-Reg pattern, consistent with our previous findings (ΔV_{30-Reg}=172±9%, p=3.0233^{-4}; $p_{30Reg-vs-NSPUni}$=0.1932). In stark contrast, neither the NSP-Beg ($\Delta V_{NSP-Beg}$=103±05%, p=0.9515) nor the NSP-End patterns ($\Delta V_{NSP-End}$=111±010%, p=0.5308) induced long-lasting structural plasticity, with the initial spine growth returning to baseline after approximately 60-75 min (Figure 2C). Importantly, the amount of structural plasticity expressed within the first 15 minutes after stimulation was not significantly different across the four paradigms (pair-wise comparisons averaged over the first 15 min and last 45 min, see Figure 2D), indicating that the differences in long lasting structural plasticity are not likely due to a stimulation failure.

Nevertheless, we considered the possibility that as some of the inter-pulse-intervals in the NSP trains were short (for example, 34 ms compared to 2000 ms

in the 30-Reg pattern), the NSP-Beg and NSP-End protocols may effectively be delivering fewer stimuli and thus less glutamate signaling, compared to their more distributed counterparts (NST-Uni and 30-Reg). In order to test whether the non-regular paradigms produce responses to each of the 30 pulses, we conducted a set of experiments in which whole-cell patch-clamp recordings were carried out during the NSP stimulations (Figure 1D). For a given pattern, we validated that each uncaging pulse produced a corresponding uncaging-mediated excitatory post-synaptic current (u-EPSC) that could be detected at the soma of the neuron as a discreet event (Figure 1D, and Supplementary Figure 3). Given that we observed this for all of the NSPs, we determined that the lack of long-term structural plasticity following NSP-Beg and NSP-End was not likely due to a reduced number of events detected by the synapse. Despite the fact that each activity paradigm contained 30 pulses and was delivered within 60s, only the 30-Reg and NSP-Uni patterns induced significant and long-lasting structural plasticity. These data suggest that the differences in the temporal structures of the stimulation patterns directly contributes to the induction of long lasting structural plasticity.

Structural plasticity induced by a naturalistic pattern (NSP-Uni) is NMDAR Dependent and Requires Protein Synthesis

The regular stimulation of a spine by glutamate uncaging has previously been shown to induce NMDAR-dependent LTP (Matsuzaki et al., 2004; Govindarajan et al., 2011). To determine whether plasticity induced by a naturalistic pattern also requires the activation of NMDA receptors, we stimulated spines with the NSP-Uni pattern in the presence of a selective NMDAR antagonist, (2R)-amino-5-phosphonopentanoate (APV), in the uncaging solution. We found that this manipulation blocked the induction of plasticity, eliciting only a transient potentiation that was not significantly different from baseline ($\Delta V_{NSP\text{-}Uni\text{-}}$

$_{APV}$=100±14%, p=0.8224, averaged over last 15 min, Supplementary Figure 4A) and significantly lower than control spines (p=0.038, compared to no APV NSP-Uni stimulation) (Figure 2E left panel). In order recruit NMDA receptor function, all of the uncaging experiments described thus far were conducted in the absence of extracellular Mg^{2+}, rather than by depolarization via whole cell patch clamping, since the latter would lead to washout of plasticity-related proteins required for long-term changes (see Kruijssen and Wierenga, 2019). To further validate the requirement for NMDA receptor activation by the NSP-Uni pattern, we also conducted experiments in which we stimulated spines in the presence of 0.25 mM extracellular Mg^{2+}. This partial blockade of NMDARs did not allow for the induction of structural plasticity ($\Delta V_{NSP\text{-}Uni\text{-}Mg}$=101±17%; p=0.8040, average of the last 45 min) (Fig 2E and Supplementary Figure 4B), and was significantly lower than that observed with control conditions (p=0.0223, compared to 0 mM Mg^{2+}) (Figure 2E right panel).

Long lasting functional plasticity that induces new protein synthesis leads to long lasting structural changes (Govindarajan et al., 2011). As we observed that the plasticity elicited at single spines with the NSP-Uni pattern leads to structural changes that last for many hours, we hypothesized that this process is also protein synthesis dependent, like its regular counterpart. Therefore, we performed both the 30-Reg and NSP-Uni stimulations in the presence of a protein synthesis inhibitor, either Anisomycin (ANI) or Cycloheximide (CHX), and evaluated the resulting changes. We confirmed that the late-phase of structural plasticity was abolished when protein synthesis was inhibited during the induction of plasticity with the Regular pattern ($\Delta V_{30\text{-}Reg\text{-}ANI}$=109±15%, p=0.6695, average of the last 45 min, compared to unstimulated controls; $\Delta V_{30\text{-}Reg\text{-}CHX}$=124±22%, p=0.6125) (Figure 2F left panel and Supplementary Figure 4C-D), in agreement with previous observations (Govindarajan et al., 2011). We found a similar blockade of structural plasticity induced by the naturalistic pattern, NSP-Uni, irrespective of the drug utilized to inhibit protein synthesis ($\Delta V_{NSP\text{-}Uni\text{-}}$

$_{ANI}$=107±9.3%, p=0.4990; $\Delta V_{NSP\text{-}Uni\text{-}CHX}$=119±1%, p=0.1476) (Figure 2F right panel and Supplementary Figure 4E-F). Thus, we find that the structural plasticity that is induced by a naturalistic pattern (NSP-Uni) at a single input is both NMDAR and protein synthesis dependent, matching the qualities of functional and structural plasticity that have been previously described.

Rapid Structural Spine Growth During Naturalistic Stimulation Predicts the Longevity of the Resulting Structural Plasticity

During the induction of glutamate uncaging mediated plasticity, a rapid initial increase in spine volume occurs (Matsuzaki et al., 2004; Harvey and Svoboda, 2007; Kruijssen and Wierenga, 2019). We wanted to determine whether similar rapid changes are triggered during naturalistic patterns of activity at individual spines. In order to visualize the temporal dynamics of spine growth that occurs during stimulation, we collected two-photon images of dendritic spines during the 60s period of glutamate uncaging for each paradigm. We tracked the growth of individual spines, and quantified the area under the curve to determine volume changes (see Supplementary Figure 5A). We found that on average, all four stimulation conditions (30-Reg, NSP-Uni, NSP-Beg, NSP-End) induced similar levels of structural plasticity during the 60 second stimulation ($\Delta V_{30\text{-}Reg}$=226±37%; $\Delta V_{NSP\text{-}Uni}$=190±19%; $\Delta V_{NSP\text{-}Beg}$=195±19:8%; $\Delta V_{NSP\text{-}End}$=236±37%, at 60s) (Figure 3B left panel) that were not significantly different from each other (p=0.7806, repeated-measures ANOVA with lower-bound adjustment, Figure 3B right panel). We noticed that in general, there was a considerable degree of variability in the amount of structural plasticity expressed by different spines during the stimulation, with some spines showing low volume changes compared to others that grew considerably, even in response to the same stimulation pattern (Figure 3C). We wondered whether these differences in short term dynamics could be predictive of long term structural plasticity outcomes of individual spines. As our three naturalistic paradigms (NSP-Uni, NSP-Beg, NSP-End) came from the same

distribution, we pooled their rapid growth dynamics and clustered them into two groups using a k-means algorithm (Bishop, 2007) (Figure 3D). The obtained clusters were termed *High* and *Low*, reflecting differences in total growth during the initial 60s of stimulus delivery. Accordingly, we plotted the corresponding long-term volume changes based on these clusters (Figure 3E left panel). We found that spines that grew more during the stimulation (in the *High* cluster) exhibited long lasting structural plasticity (ΔV_{High}=158±20%, average of last 45 min), while spines that showed little growth (in the *Low* cluster) exhibit only short lived volume changes (ΔV_{Low}=113±7%, Figure 3E right panel). We also found that a second analysis, in which the data were fit to a bimodal distribution, showed similar results in which two groups of low and high growers predicted which spines underwent long term structural changes based on their rapid growth dynamics (Supplementary Figure 5). Therefore, while different activity patterns led to clear differences in long-term structural plasticity, the short-term dynamics revealed that a higher diversity of responses existed among the population of individual spines to these stimuli. Further, we found that the degree to which any given spine grows over the course of naturalistic activity is highly predictive of the structural plasticity that it will subsequently express.

Longevity of structural plasticity also varies with different stationary patterns, yet is not correlated with short-term spine dynamics.

We wondered what aspect of the pulse distribution among the different protocols was leading to the differences in the longevity of structural plasticity that was elicited. We considered the possibility that the consistent delivery of stimuli over the entirety of the 60s window, rather than the number of stimuli per se, was the critical parameter to the induction of strong plasticity. In addition, it was unknown whether the closely spaced stimuli that occurred at various intervals in the NSP patterns would contribute to the induction of plasticity with the same efficacy as events that are more evenly distributed during the activity pattern. To test these

possibilities, we designed two alternative stimulation patterns that were stationary in nature and that would be delivered over the same 60 sec period as our 30 pulse protocol (30-Reg, Figure 4A).

We first stimulated individual spines with a lower frequency stimulation that was nevertheless regular in nature, by delivering 15 equally spaced pulses (15-Reg: at 0.25Hz). The 15-Reg pattern only induced weak growth, which by 60-75min after the stimulation was not significantly different than baseline ($\Delta V_{15\text{-}Reg}$=137±15%, p=0.2844, average over last 45min, compared to neighbors) (Figure 4B). This is similar to the length for which structural changes lasted with both NSP-Beg and NSP-End. This finding indicates that a regular pattern alone is not sufficient to induce long lasting structural plasticity.

We next wanted to determine how the inter-pulse-interval impacted the induction of structural plasticity, as this varied in our NSP paradigms. We delivered 15 pairs of pulses (15-Paired: 15 pairs of pulses spaced 50 ms apart, delivering a total of 30 pulses at 0.5Hz) to test whether 30 stimulations, in which half occurred in closely spaced temporal proximity to one another (50ms), would give rise to structural plasticity of similar magnitude to that induced with evenly distributed events. We found that the 15-Paired protocol produced long lasting structural plasticity ($\Delta V_{15\text{-paired}}$ =187±23%, p=0.0027, average of the last 45 min, compared to neighbors) (Figure 4C left panel) that was similar in magnitude to what we observed with the 30-Reg paradigm (p=0.6126, average of the last 45min) (Figure 4C right panel). The fact that the 15-Paired protocol yielded long lasting structural plasticity while the 15-Reg did not, suggests that each pulse likely contributes toward the induction of plasticity, including those in close temporal proximity, in accordance with our initial observations (Figure 1D). Together, these data indicate that a minimal quantity of stimulation is required in order to achieve long lasting structural plasticity, and that the regularity of a pattern, in and of itself, is insufficient to accomplish this.

As we had observed variability in the amount of spine volume growth during the stimulations with the naturalistic patterns, we wondered whether similar spine growth dynamics would be observed during stimulation with the different stationary paradigms (30-Reg, 15-Reg, 15-Paired) (Figure 4D). We wanted to know if the short term volume changes were predictive of the long term structural plasticity outcomes, similarly to what we had observed with NSPs. Spines stimulated with regular stimulation patterns did indeed produce a range of responses that could be classified into *Low* and *High* growing groups, similar to that observed with NSP stimulations (Figure 4D and 4E). However, unlike the case of NSPs, *Low* and *High* growing spine responses during stationary patterns of activity did not correlate with divergent long term structural outcomes ((ΔV_{Low}=163±18%, ΔV_{High}=159±14%, p=0.7618, average of the last 45 min) (Figure 4F). This observation is in stark contrast with the naturalistic paradigms whose short term spine dynamics predicted the longevity of plasticity.

Discussion

Synaptic plasticity has been widely studied through the delivery of regular trains of activity using frequencies sampled from *in vivo* recordings, but they have much less often reproduced the irregularities of inter-spike intervals that are common during endogenous activity (Zador and Dobrunz, 1999; Dobrunz and Stevens, 1999; Paulsen and Sejnowski, 2000). It is unknown whether such irregular patterns can induce plasticity at single inputs and what if any would be the structural consequences of this form of activity. Here, using two-photon fluorescence imaging and glutamate uncaging, we studied single spine structural plasticity of CA1 pyramidal neurons using stimulation patterns sampled from a Poisson process, resembling firing patterns of CA3 neurons. For the first time, we have shown that the longevity of this plasticity is determined by the timing structure of NSPs. In addition, we show that rapid structural changes induced by NSPs, but not by stationary patterns, predict the longevity of the induced

changes. These findings suggest that a single dendritic spine has the capacity to integrate temporal information with millisecond resolution, that the result of this transformation can modulate the longevity of plasticity that is expressed, and that the rapid growth of the spine in response to stimuli can predict whether that activity was sufficiently salient to induce structural plasticity.

In our experiments, NSP-Uni was the only naturalistic pattern that induced long-lasting structural changes. This finding was surprising, given that each of the naturalistic activity patterns delivered the same total amount of glutamate over the same period to each spine (30 pulses over 60 s). Nevertheless, the pattern of activity delivered in that first minute influenced the structural plasticity that was expressed over subsequent hours. What is the critical difference between these? Although sampled from the same Poisson distribution, NSP-Beg and NSP-End had instantaneous frequencies over time that were considerably different than NSP-Uni, which was the only pattern to induce long-lasting plasticity and was the most similar in the distribution of events to that of the 30-Reg (see Supplementary Figure 2). These time-frequency structures may influence Ca^{2+} concentrations, and thus impact the signaling pathways that are recruited during the induction of plasticity (Cooper et al., 2012). Therefore, stationarity seems to be an important component that leads to the induction of long-lasting structural plasticity, despite the fact that it may not be sufficient to induce plasticity if a minimum stimulation threshold is not achieved. We find that with half the number of stimulations of our standard protocol (15-Reg vs 30-Reg), regularity itself is not sufficient to induce plasticity (Fig 4B). This is in agreement with previous findings in which 1 ms-long, rather than 4 ms long, regular uncaging pulses do not induce structural changes (Harvey and Svoboda, 2007; Govindarajan et al., 2011).

The differential activation of several key molecular players may contribute to the selective induction of plasticity with the NSP-Uni paradigm. A recent study using computational modeling showed that the extracellular signal-regulated kinase (ERK) is sensitive to temporal patterns of synaptic activation (Blackwell et al.,

2021). Also, activation of Calcium/calmodulin kinase II (CaMKII), crucial for the induction of LTP, and has been shown to be sensitive to the overall number and frequency of stimuli, as well as be responsive to activity pattern dependent subunit regulation (Lee et al., 2009; Fujii et al., 2013; Singh and Bhalla, 2018). Thus, the different patterns of activity delivered by our NSP protocols may differentially activate key signaling components.

We found that NSP-Uni requires NMDA-R activation and new protein synthesis, thereby utilizing the canonical pathways involved in the induction and maintenance of synaptic functional and structural plasticity. Importantly, our findings demonstrate that synaptic activity patterns can differentially induce long lasting and protein synthesis dependent structural plasticity. The late phase of LTP as well as long lasting structural plasticity with regular patterns requires newly synthesized proteins (Otmakhova et al., 2000; Sutton and Schumann, 2006; Govindarajan and Israely et al., 2011). Our results demonstrate that naturalistic patterns of activity induce similar mechanisms at individual spines, and it will be important to determine what are the temporal dynamics and their targets which regulate the function of these critical plasticity pathways.

One consequence of activity through NMDARs, necessary for the induction of functional and structural plasticity, is postsynaptic calcium entry. Differential concentrations of Ca^{2+} have been proposed as mechanisms for establishing bidirectional changes in plasticity, via distinct activation of kinase or phosphatase driven pathways (Otmakhov et al. 1997; Lisman and Spruston, 2005; Asrican et al., 2007; Cooper et al., 2012). It is therefore possible that a given stimulation pattern, and in particular the micro-domains within that pattern, could dynamically modulate the relative balance of active signaling components necessary for determining the expression of structural plasticity, and it will be relevant to examine whether alternative NSP patterns can induce bidirectional forms of structural plasticity.

It had previously been shown that dendritic spine structure can change rapidly upon activation (Van Herreveld and Fifkova, 1975; Fifkova, 1985; Harvey et al., 2008; Lee et al., 2009). When we examined the rapid structural responses of spines during different stimulation paradigms, we did not see significant differences between their responses to the four different conditions. However, when we investigated individual structural growth traces, we noticed variability across spines. Clustering analyses revealed that the relationship between short- and long-term dynamics is stimulation pattern dependent for naturalistic patterns. On the other hand, no such relationship was observed with regular patterns of activity. These findings suggest that there is a difference in the saliency of the naturalistic patterns compared to that of stationary activity at individual spines, and it will be important to identify what are the critical features of non-stationary patterns of activity and the inputs that they engage, which gives rise to such variabilities in structural plasticity outcomes. Further, how these short-term temporal dynamics engage downstream signaling processes, such as protein synthesis dependent mechanisms, remains to be determined.

Here, we present the first experimental evidence for how different temporal patterns of statistically similar activity can elicit different longevities of structural plasticity at single dendritic spines. The structural plasticity that is induced by these Poisson patterns acts through canonical plasticity mechanisms, recruiting NMDA receptor function and inducing new protein synthesis. Of interest, we observe a diversity of responses across individual stimulated spines, which is predictive of their subsequent long term structural changes. These findings highlight the fact that diverse patterns of endogenous activity can give rise to various degrees of structural plasticity and begins to elucidate the learning rules associated with naturalistic activity patterns at individual spines, the fundamental inputs of neuronal activity.

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Author Contributions

A.Ö.A and I.I. designed the experiments. A.Ö.A performed the experiments, analyzed the data and drafted the manuscript. I.I. supervised the study and wrote the manuscript.

Declaration of Interest

The authors declare no competing interests.

Figure Legends

Figure 1. Generation and Delivery of Naturalistic Stimulation Patterns at Single Dendritic Spines.

(A) Artificially generated Poisson patterns with a λ=30 and the respective Inter Pulse Interval distributions (IPI) used to simulate naturalistic activity. Dashed red line represents the average IPI interval of the distribution, which is also the IPI for the 30-Reg pattern (2s). Only 740 out of 10000 generated patterns had exactly 30 events in 60s (inset).

(B) Schematic representation of the experimental flow. Four glutamate uncaging patterns used in the study. Each pulse within the stimulation pattern is represented by a thin bar, and all pulses are delivered within a 60 sec period. The 30-Reg (Black) pattern has 30 pulses in 60s with a fixed IPI of 2s. The remaining three patterns were picked from the 740 generated patterns that contained exactly 30 events. Patterns were sorted according to the number of events distributed across three 20s time bins. NSP-Uni (Blue) has 10 pulses every 20s, NSP-Beg (Brown) has 15 pulses in the first 20s bin and 15 pulses in the last 40s, NSP-End (Green) has 15 pulses in the first 40s and 15 pulses in last 20s. Each pattern was delivered in 60 seconds to a single spine via 2-photon uncaging of MNI-glutamate in ACSF containing 0mM Mg^{2+} that was introduced via a re-circulation system. Z-stacks of the dendritic branch of interest were imaged every 5 minutes before (30 min. baseline) and after (~210 min. post stimulation) uncaging mediated stimulation.

(C) Mouse organotypic hippocampal slice cultures were transfected by gene gun to sparsely label CA1 pyramidal neurons with GFP, and fluorescent neurons were imaged using 2-photon microscopy. In some experiments whole cell patch clamp recordings at the cell soma were performed to monitor synaptic responses during uncaging stimuli.

(D) Temporal efficacy of responses to individual glutamate uncaging pulses was confirmed with simultaneous uncaging and whole cell patch clamp recordings. A representative example from an experiment in which the NSP-Beg pattern was used to stimulate a spine. Pink and blue boxes indicate regions expanded on the right, showing that individual synaptic events that are delivered at either 120ms or 34ms respectively, can be detected as separate synaptic events.

Figure 2. The Temporal Dynamics of Uncaging Patterns Determine the Longevity of Single Spine Plasticity.

(A) A 2-photon image of a biolistically transfected CA1 pyramidal neuron in which a single spine undergoes uncaging mediated stimulation. The red dot represents the uncaging point (720nm 2-photon laser). The yellow box represents the dendritic region of interest (ROI) that was imaged, and the red box represents a zoomed in view of the stimulated spine ROI. Scale bar is 2μm.

(B) A representative uncaging experiment. The upper panel shows the stimulated spine (red triangle) and neighboring spines before (left) and after (right) uncaging stimulation. The lower panel shows the automatic segmentations of spine heads using the SpineS toolbox for volume quantification. The spine was stimulated at time 0 min. Scale bar is 2μm.

(C) Spine volume changes over 2 hours in response to glutamate uncaging mediated stimulation with different patterns of activity. Spine volumes are quantified for stimulated and neighboring spine volumes during all four stimulation conditions, and are represented as mean ± SEM. The 30-Reg pattern (in black) induced structural plasticity that lasted 3.5 h (n_{stim} = 14, n_{neigh} = 176, N=11 animals). NSP-Uni (in blue) induced long lasting plasticity that was similar to the 30-Reg pattern (n_{stim} = 13, n_{neigh} = 155, N=8 animals). NSP-Beg (in brown) induced only short lasting changes (n_{stim} = 17, n_{neigh} = 202, N=8 animals). NSP-End (in green) induced short lasting structural changes (n_{stim} = 18, n_{neigh} = 264,

N=9 animals). n represents number of neurons each from an independent slice, while N is number of animals.

(D) All patterns induce structural plasticity within the first minutes following stimulation, however those induced by NSP-Beg and NSP-End were not long lasting, and returned to baseline by 60 min post stimulation (Mann-Whitney U). The average volume change observed during the first 15 min as well as the last 45 min are compared across stimulation paradigms.

(E) NSP-Uni mediated structural plasticity requires NMDA receptor activation, and is inhibited with either pharmacological blockade of NMDA receptors using APV or with partial magnesium blockade of those receptors (APV: n_{stim} = 5, n_{neigh} = 73; Mg: n_{stim} = 4, n_{neigh} = 44).

(F) The late phase of NSP-Uni induced structural plasticity is protein synthesis dependent. Open bars represent neighboring spine volumes, while solid bars represent volume of the stimulated spines. Stimulated spines did not grow in response to activity in the presence of Anisomycin or Cycloheximide. Bars represent mean ± SEM (30-Reg: Aniso: n_{stim} = 10, n_{neigh} = 169; Cyclo: n_{stim} = 6, n_{neigh} = 85; $p_{ani_vs_cyc}$ = 0.6354. NSP-Uni: Aniso: n_{stim} = 9, n_{neigh} = 126; Cyclo: n_{stim} = 5, n_{neigh} = 72; $p_{ani_vs_cyc}$ = 0.4970, unstimulated controls vs stimulated spines, average over last 45', Mann-Whitney U).

Figure 3. Rapid Spine Growth During the Stimulation Predicts the Longevity of Structural Plasticity Induced by Naturalistic Patterns.

(A) Rapid structural growth is observed during the 60 seconds of glutamate uncaging mediated stimulation. The upper panel shows the dendrite of interest and the stimulated spine is contained within the yellow box. The lower panels show spine structural growth over the course of the stimulation (in seconds). Scale bar is 2μm.

(B) Statistical comparison of the growth data for each stimulation condition reveals no difference between the growth curves (Repeated Measures ANOVA) of any of the naturalistic patterns.

(C) Rapid growth dynamic trajectories for all of the individual spines stimulated with the respective NSP pattern Uni, Beg, End (Blue, Brown and Green) ($n_{NSP-Uni}$=13, $n_{NSP-Beg}$=17, $n_{NSP-End}$=18, n_{High}=16 ,n_{Low}=32).

(D) Pooled growth curves from each of the three NSP patterns. Rapid growth curves were divided into two classes using a k-means algorithm and color coded accordingly (*High* growers: red, *Low* growers black). Numbers in the adjacent pie charts show the contribution of each condition to that particular cluster.

(E) Long term spine volume changes were plotted according to the k-means identified grouping of high (red) or low growers (black), demonstrating a correspondence between the rapid structural changes and the long lasting ones. This is quantified in the bar graphs on the right, showing that while both clusters induced similar levels of initial structural plasticity (at 15'), the longevity of plasticity significantly diverges, as seen at time bins around 100' and 225' ($p_{0-15'}$=0.0646, $p_{90-120'}$=7.9883e-4, $p_{180-225'}$=0.0086, Mann-Whitney U)

Figure 4. Rapid Spine Growth with Stationary Uncaging Patterns does not Predict the Longevity of Structural Plasticity.

(A) Schematic of the stationary stimulation patterns used to induce structural plasticity at single spines. In addition to the 30-Reg (black) pattern (30 pulses at 0.5 Hz), we also tested a pattern that delivered half the number of pulses in the same time (15-Reg, 0.25 Hz, yellow) and a pattern that delivered the same 30 pulses in the form of 15 pairs with a 50 ms inter-pulse-interval (15-Paired, magenta).

(B) Spine structural plasticity as measured by volume increases following stimulation with either the 15-Reg (yellow) pattern or the 15-Paired (magenta)

pattern. The 15-Reg pattern shows only short term structural changes, while the 15-Paired stimulation produces robust long lasting structural plasticity.

(C) The 15-Paired pattern (magenta) induced long lasting structural changes whereas the 15-Reg (yellow) only gave rise to short term structural changes. The 15-Paired stimulation induced higher levels of plasticity in the first 120' compared to 30-Reg that leveled afterwards. Spine volumes were quantified in three different bins 0-15', 90-120' and last 45'. Comparisons were made with corresponding unstimulated neighbors and between conditions using Mann-Whitney U.

(D) Rapid growth dynamics during the stimulation with either the 15-Reg, 15-Paired or 30-Reg stimulations show that growth trends are variable across individual spines as during NSP stimulations.

(E) Rapid spine growth trajectories during the stimulations of 30-Reg, 15-Reg, and 15-Paired ($n_{15\text{-Reg}}$=8, $n_{15\text{-Paired}}$=8, $n_{30\text{-Reg}}$=10) were pooled. Numbers in the adjacent pie charts show contribution per condition to each group, obtained by k-means cluster analysis.

(F) Short-term structural plasticity groups were evaluated for their correlation with long term structural outcomes, and graphed according to the rapid growth clusters identified by k-means analysis. Both groups induced equal amounts of structural plasticity ($p_{0\text{-}15'}$=0.6597, $p_{90\text{-}120'}$=0.9805, $p_{180\text{-}225'}$=1, Mann-Whitney U).

Supplementary Figure 1. Uncaging Evoked EPSCs During 1-Minute-Long Uncaging Delivery

(A) The uncaging patterns used in the delivery of the 30-Reg, NSP-Uni, NSP-Beg and NSP-End stimulations.

(B) Table with inter pulse intervals for the different stimulation paradigms: 30-Reg, NSP-Uni, NSP-Beg and NSP-End,.

(C) Instantaneous pulse frequencies of delivered uncaging patterns. 30-Reg has a constant frequency of 0.5 Hz (black solid line), NSP-Uni pattern has instantaneous frequencies fluctuating around 0.5 Hz (blue), NSP-Beg pattern has a higher instantaneous frequency in the first 20 sec (brown) that drops later in the stimulation period, and NSP-End has a higher instantaneous frequency during the last 20 sec (green). Red represents the instantaneous frequencies of all generated Naturalistic-like patterns combined.

Supplementary Figure 2. Individual spine volume traces for all condition

All stimulated spine volume trends are shown for each of the four conditions tested. Bold lines represent group means. ($n_{30\text{-}Reg}$ = 14, N=11 Litters; $n_{NSP\text{-}Uni}$ = 13, N=8 Litters; $n_{NSP\text{-}Beg}$ = 17, N=8 Litters; $n_{NSP\text{-}End}$ = 18, N=9 Litters). n is number of neurons, N is number of animals. Only one dendritic spine is stimulated per neuron. Hence, the number of spines are equal to the number of neurons.

Supplementary Figure 3. Uncaging Evoked EPSCs (uEPSCs) during Stimulations with Different Naturalistic Patterns

For each pattern, 4 or 5 independent spines were stimulated using glutamate uncaging and the resulting uncaging evoked excitatory post-synaptic currents (u-EPSCs) were recorded via whole-cell patch clamp electrophysiology. 30 uncaging pulses were delivered per condition, according to the stimulation patterns described earlier. uEPSCs are plotted as the mean of 5 consecutive responses in a non-overlapping fashion (i.e. for 30 pulses in total, the average of

the first five responses is shown in the black trace, the average of the last five responses is shown in red.) Gradient represents intermediate response averages.

Supplementary Figure 4. Spine structural plasticity following blockade of NMDA function or new protein synthesis.

(A) NMDA blockade inhibits the expression of long lasting structural plasticity. Time course of NSP-Uni stimulation in the presence of APV (n_{stim} = 5, n_{neigh} = 73)(solid aqua).

(B) Partial inhibition of NMDA function in low magnesium (0.25 mM) significantly reduces the expression of long lasting structural plasticity. Time course of NSP-Uni stimulation in the presence of 0.25mM Mg (n_{stim} = 4, n_{neigh} = 44)(blue line).

(C) Protein synthesis inhibition shortens the expression of long lasting structural plasticity. 30-Reg stimulation during protein synthesis blockade with Anisomycin (n_{stim} = 10, n_{neigh} = 169)(solid gray).

(D) Protein synthesis inhibition shortens the expression of long lasting structural plasticity. 30-Reg stimulation during protein synthesis blockade with another protein synthesis inhibitor, Cycloheximide (n_{stim} = 6, n_{neigh} = 85)(solid pink).

(E) Protein synthesis inhibition shortens the expression of long lasting structural plasticity during NSP-Uni stimulation as well, here using the protein synthesis inhibitor Anisomycin (n_{stim} = 8, n_{neigh} = 114).

(F) Again, protein synthesis inhibition shortens the expression of long lasting structural plasticity during NSP-Uni stimulation, using a second protein synthesis inhibitor, Cycloheximide (n_{stim} = 4, n_{neigh} = 63).

Supplementary Figure 5. Secondary Analysis for the Evaluation of the Predictive Capacity of Rapid Spine Dynamics Induced by Naturalistic Patterns

(A) We used the area under the curve (AUC, or integral) for each spine during the period of rapid growth upon stimulation, and used this to fit a 1-dimensional

Bimodal Gaussian. We used the resulting two groups identified in this bi-modal distribution to identify two clusters of high and low growers during uncaging stimulations. We wanted to check that a secondary analysis, here using a Gaussian fit, gave us similar results to the k-means clustering analysis that we conducted. Indeed, we found that the high growers identified in this way corresponded to spines that showed long lasting structural changes, while the low growers did not.

Supplementary Figure 6. Determining Spine Volume Changes via Semi-Automated Spine Shape Segmentation with the SpineS Toolbox

(A) An example of a stimulated spine that grew upon glutamate uncaging and maintained an enlarged size over time. Upper row shows the maximum intensity projection (MIP) from the 3-dimensional image stack. The lower row shows the results of the segmentation software and the accurate delineation of the spine head.

(B) Neighboring spines did not show significant, sustained changes in volume, as quantified using the SpineS software. Even spine heads located in close proximity to the dendritic branch can be segmented successfully.

STAR Methods

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Inbal Israely (ii2176@columbia.edu).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

The datasets and analysis routines are available from the corresponding author on reasonable request.

Ethics Statement

All animal experiments were carried out in accordance with European Union regulations on animal care and use, and with the approval of the Portuguese Veterinary Authority (DGV).

Method Details

Preparation of Organotypic Slice Cultures

Cultured hippocampal slices were prepared from postnatal day 7–10 C57BL/6J mice (Stoppini et al., 1991). Briefly, 350 μm thick slices were made with a chopper in ice-cold ACSF containing 2.5 mM KCl, 26 mM $NaHCO_3$, 1.15 mM NaH_2PO_4, 11 mM D-glucose, 238 mM sucrose, 1 mM $CaCl_2$ and 5 mM $MgCl_2$, and cultured on membranes (Millipore). The slices were maintained in an interface

configuration with the following media: 1× MEM (Invitrogen), 20% horse serum (Invitrogen), GlutaMAX 1 mM (Invitrogen), 27 mM D-glucose, 30 mM HEPES, 6 mM $NaHCO_3$, 1 M $CaCl_2$, 1 M $MgSO_4$, 1.2% ascorbic acid, 1 µg/ml insulin. Media was changed every 2 to 3 days. The pH was adjusted to 7.3, and osmolarity adjusted to 300–310 mOsm. All chemicals were from Sigma unless otherwise indicated.

Biolistic Gene Transfection

Slice cultures were transfected using a Helios gene gun (Bio-Rad) after 4–5 days in vitro (DIV). Gold beads (10 mg, 1.6 µm diameter, Bio-Rad) were coated with 100 µg AFP plasmid (Ogawa and Umesono, 1998) DNA according to the manufacturer's protocol and delivered biolistically into the slices at 160-200 psi. Experiments were performed 3–7 days post-transfection.

Patch Clamp Electrophysiology

Hippocampal slice cultures were pre-incubated for 45 min to 1 h at room temperature and perfused continuously with ACSF. Whole cell voltage-clamp recordings were performed in CA1 pyramidal neurons, using 7–8 MΩ electrodes filled with internal solution containing: 136.5 mM K-gluconate, 9 mM NaCl, 17.5 mM KCl, 10 mM HEPES, 0.2 mM EGTA, pH adjusted to 7.2 with KOH, and 284 mOsm. Cells were voltage clamped at −65 mV. Cellular recordings in which series resistance was higher than 25 MΩ were discarded, and stability was assessed throughout the experiment (±20%). uEPSC responses were evoked by glutamate uncaging. Signals were acquired using a Multiclamp 700B amplifier (Molecular Devices), data was digitized with a Digidata 1440 at 3 kHz. EPSC amplitudes were analyzed using custom software written in Matlab.

Generation of Naturalistic Stimulation Patterns

We generated 10000 patterns by a homogeneous Poisson process with a rate of r = 0.5 (Hz) spanning 60s by generating interspike interval patterns from a uniform distribution between 0 and 1 whose negative logarithm is exponentially distributed. Hence, we can have generated pulse times t(i) iteratively from the formula t(i+1) = t(i) - log(x)/r. (see Heeger 2000 for details).

Two photon imaging and uncaging

Two-photon imaging was performed at 910 nm using a galvanometer-based scanning system (Prairie Technologies, acquired by Bruker Corporation) on a BX61WI Olympus microscope, using a Ti:sapphire laser (Coherent Inc.) controlled by PrairieView software. Slices were perfused with oxygenated ACSF containing 127 mM NaCl, 2.5 mM KCl, 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 25 mM D-glucose, 2 mM $CaCl_2$ and 1 mM $MgCl_2$ (equilibrated with O_2 95%/CO_2 5%) at 38°C to achieve room temperature in the chamber at a rate of 1.5 ml/min. Imaging was started 45 min to 1 h after slice incubation began. Secondary or tertiary dendrites of CA1 neurons were imaged using a water immersion objective (60X, 0.9 NA, Olympus) with a digital zoom of 10X. Z-stacks (0.3 µm per section) were collected once every 5 min for up to 4 hours at a resolution of 1024×1024 pixels, resulting in a field of view approximately 20 µm × 20 µm. Imaging laser power and PMT gain settings were kept constant throughout each experiment.

To induce plasticity, uncaging patterns were applied by positioning the laser 0.5 mm from the tip of the spine head and uncaging MNI-glutamate (2.5 mM) with a stimulus pattern consisting of 4ms-long 30mW laser pulses (720nM,). Glutamate uncaging was done in the absence of extracellular Mg2+, in order to allow for the observation of long-lasting structural changes without washing out plasticity-related proteins during whole cell physiology (see Kruijssen and Wierenga, 2019). Stimulations were carried out at spines located on secondary or tertiary dendrites within stratum radiatum of CA1 pyramidal neurons sparsely labelled with GFP by gene gun (Figure 2A). The region containing the stimulated spine

and unstimulated neighbors was imaged every 5 minutes for a baseline period of 20 minutes, a selected spine underwent uncaging stimulation for 60s, and the dendritic region was subsequently imaged every 5 minutes for up to 2 hours (Figure 2A).

Data Analysis

All analysis were performed using Matlab (MathWorks). We quantified changes in spine volume using a Matlab based toolbox that we developed, called SpineS, in which the semi-automatic segmentation of spine heads occurs in an unbiased manner (Erdil et al., 2012; Rada et al., 2014; Rada et al., 2018; Argunsah et al., 2020) (Figure 2B, Supplementary Figure 5). All normalization was performed on a per spine basis as a percent of the average baseline value for that spine. We imaged dendrite of interest every 5 minutes and analyzed in 15min bins. Results are presented as mean ± SEM.

Statistics

All statistical analyses were performed using custom code written in Matlab (MathWorks). Nonparametric Mann-Whitney U test was used to compare spine volumes at any time bin versus baseline or different condition. Time series were compared using repeated-measures ANOVA. Stars (*) represent degrees of significance as follows: (*) = $p < 0.05$; (**) = $p < 0.01$; (***) = $p < 0.001$).

KEY RESOURCE TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals		
MNI-caged-L-glutamate	TOCRIS	CAS No: 295325-62-1; Batch No: 40A/160038, 36A/147239, 35A/140571,

		32A/127489, 32A/133545, 43A/174380, 40A/165397
Cycloheximide	TOCRIS	CAS No: 66-81-9
Anisomycin	TOCRIS	CAS No: 22862-76-6; Batch No: 9A/172390, 7A/144486
APV	TOCRIS	CAS No: 79055-68-8
Tetrodotoxin citrate (TTX)	TOCRIS	CAS No: 18660-81-6 Batch No: 43C, 43b, 45A
Software and Algorithms		
Ultima In Vitro Multiphoton Microscope System	Bruker	https://www.bruker.com/products/fluorescence-microscopes/ultima-multiphoton-microscopy/ultima-in-vitro/overview.html ; RRID:SCR_017142
Matlab	MathWorks	http://www.mathworks.com/products/matlab/ ; RRID:SCR_001622
pClamp 10	Molecular Devices	http://www.moleculardevices.com/products/software/pclamp.html/ ; RRID:SCR_011323
Dendritic spine segmentation and analysis	Argunsah et al., 2020.	https://github.com/argunsah/SpineS

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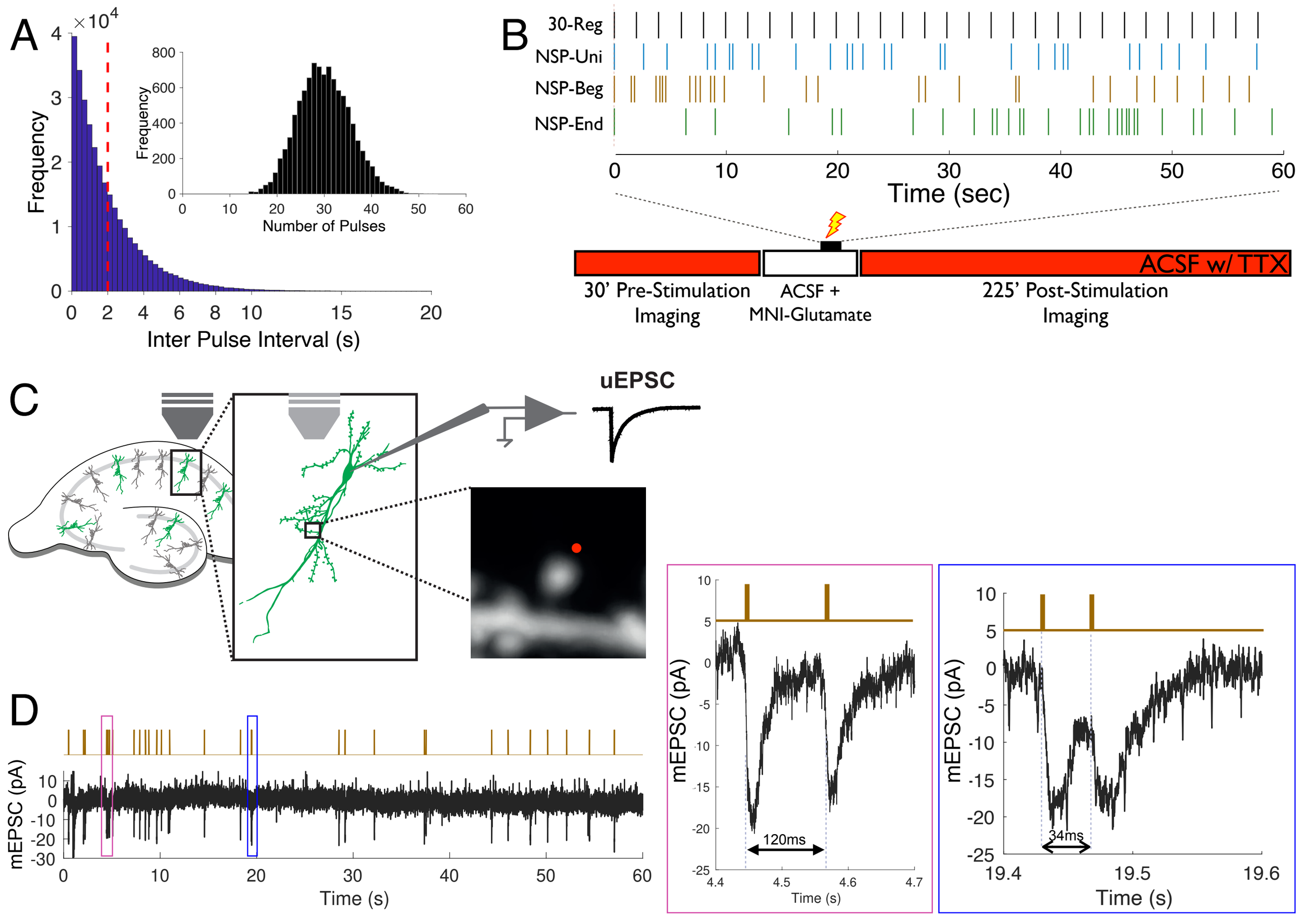
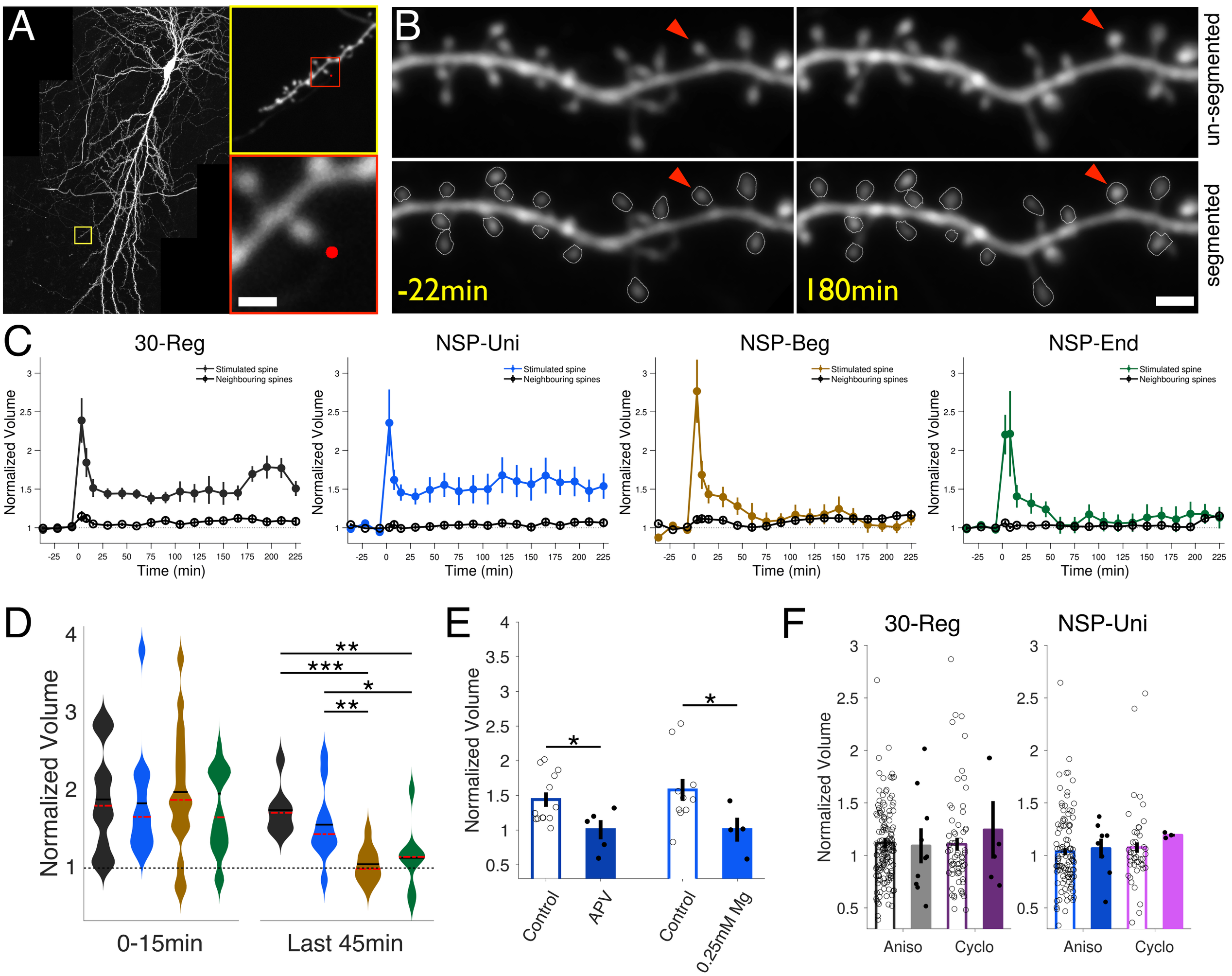


Figure 1 - Argunsah and Israely, 2021.



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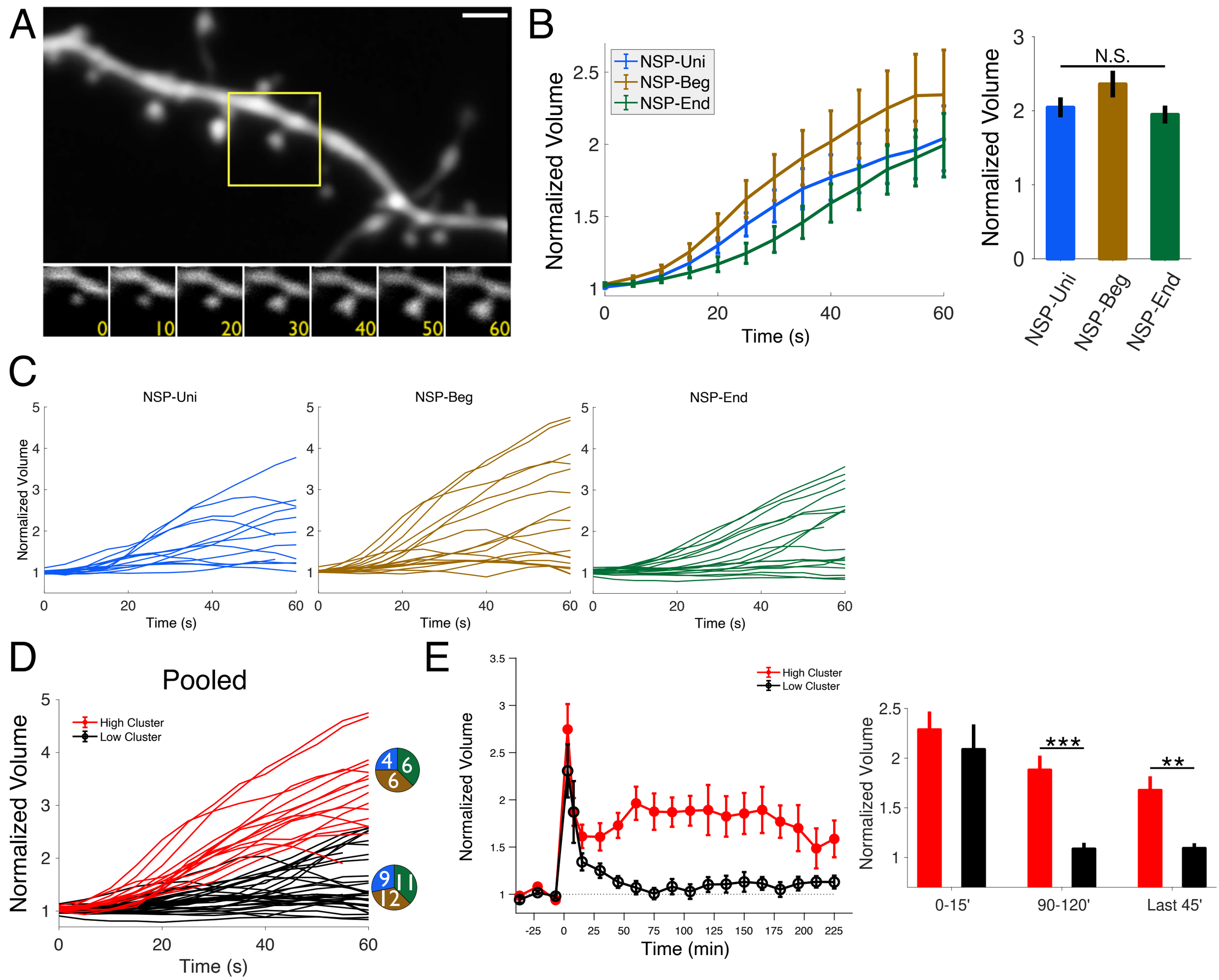
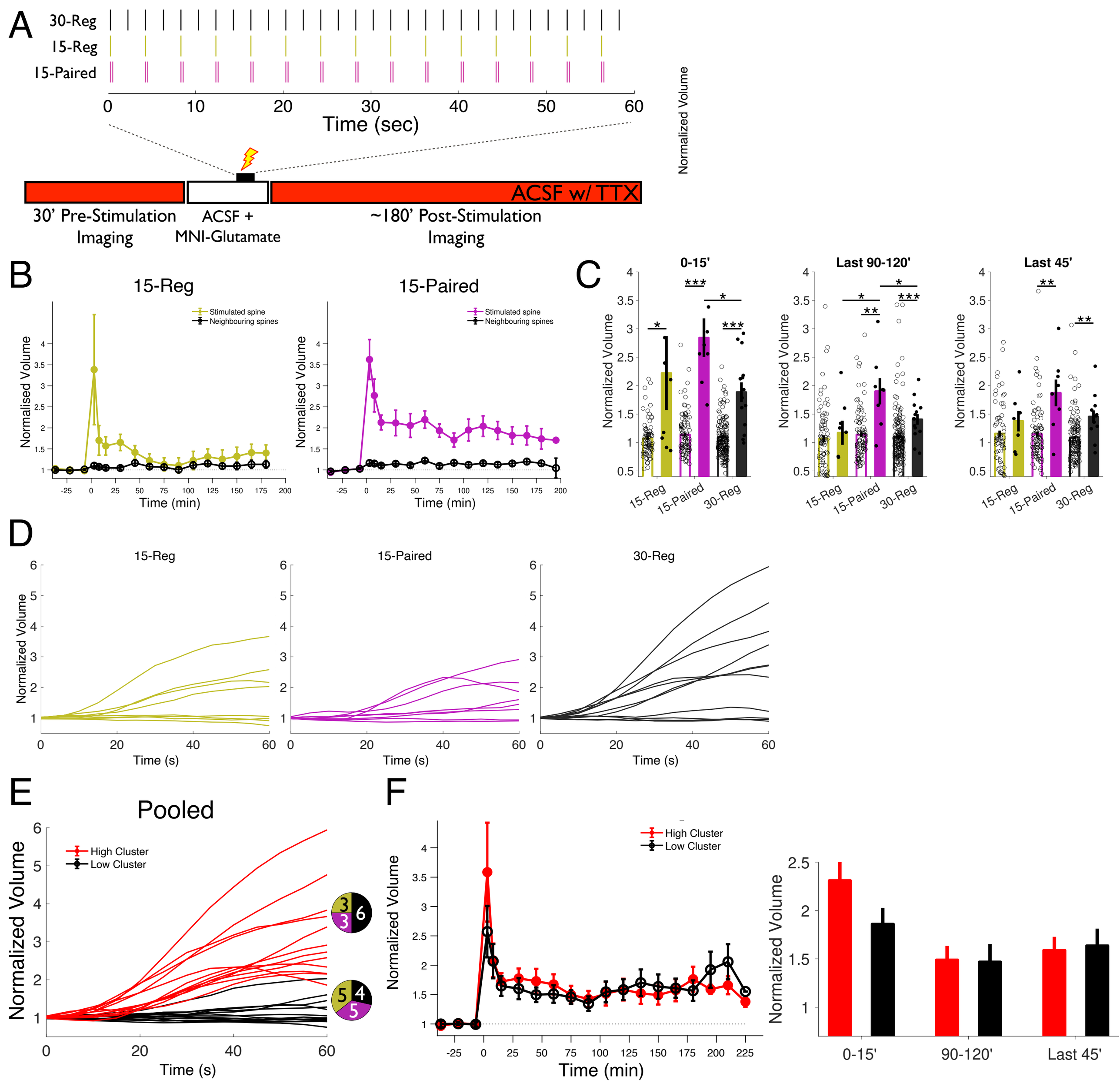


Figure 3 - Argunsah and Israely, 2021.



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