Divergent scaling of miniature excitatory post-synaptic current amplitudes in homeostatic plasticity

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DIVERGENT SCALING OF MINIATURE EXCITATORY POST-SYNAPTIC CURRENT AMPLITUDES IN HOMEOSTATIC PLASTICITY

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

by

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ABSTRACT

Hanes, Amanda L. Ph.D. Biomedical Sciences PhD Program, Wright State University, 2018. Divergent scaling of miniature excitatory post-synaptic current amplitudes in homeostatic plasticity

Synaptic plasticity, the ability of neurons to modulate their inputs in response to changing stimuli, occurs in two forms which have opposing effects on synaptic physiology. Hebbian plasticity induces rapid, persistent changes at individual synapses in a positive feedback manner. Homeostatic plasticity is a negative feedback effect that responds to chronic changes in network activity by inducing opposing, network-wide changes in synaptic strength and restoring activity to its original level. The changes in synaptic strength can be measured as changes in the amplitudes of miniature post-synaptic excitatory currents (mEPSCs). Together, the two forms of plasticity underpin nervous system functions such as movement, learning and memory, and perception, while preventing pathological states of hyper- or hypoactivity that could occur if network activity were not maintained. The current hypothesis of homeostatic plasticity states that mEPSC amplitudes exhibit uniform multiplicative scaling, a transformation in which the amplitudes are scaled up or down globally by a multiplicative factor. This hypothesis constrains the possible mechanism of homeostatic plasticity, which remains unknown despite intensive study.
Here, we compare an experimental data set previously collected in our laboratory to the results of an empirical simulation of uniform multiplicative scaling and conclude that the homeostatic increase in mEPSC amplitudes in our data is not uniform. We develop and validate a novel method, comparative standardization, for calculating the scaling transformation between treated and untreated mEPSC amplitudes and identifying the transformation as either uniform, divergent, or convergent. When applied to our experimental data, comparative standardization finds divergent scaling, in which the homeostatic effect increases with synaptic strength, causing the control and treated mEPSC amplitude distributions to diverge. The divergent scaling transformation computed by comparative standardization is also more accurate than the transformations computed by existing methods. Finally, we generalize our findings by applying our approach to several additional homeostatic plasticity data sets obtained from our collaborators: All additional data exhibit divergent scaling, and comparative standardization consistently outperforms both existing methods for computing the homeostatic scaling transformation.
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I. INTRODUCTION

Synaptic transmission and long-term plasticity

The function of the central nervous system is based upon the ability of neurons to form networks capable of storing and processing information through the modulation of intercellular connections. Synaptic transmission is the process by which information, in the form of a chemical signal, is passed from one neuron to another at a specialized site called a synapse. An action potential reaching the nerve terminal of the presynaptic neuron depolarizes the cell membrane, activating voltage-gated calcium channels that allow calcium ions to enter the cell. This raises the concentration of intracellular calcium, which causes vesicles containing neurotransmitter molecules to fuse with the cell membrane and release the neurotransmitter into the synaptic cleft. The neurotransmitter diffuses across the synaptic cleft and activates ligand-gated ion channels on the postsynaptic membrane, resulting in a post-synaptic current that can depolarize or hyperpolarize the cell, depending on the reversal potential of the ions to which the channel is permeable. A depolarizing current is called an excitatory post-synaptic current (EPSC) because it usually contributes to the generation of action potentials, and a hyperpolarizing current is called an inhibitory post-synaptic current (IPSC). In addition to the synchronous fusion of multiple vesicles in response to an action potential, single vesicles can exocytose spontaneously, resulting in a miniature EPSC (mEPSC) or IPSC. The amplitude of a post-synaptic current or miniature post-synaptic current is the functional determinant of synaptic strength, as a larger current is more likely to evoke a response in the post-synaptic neuron.
The ability to effectively modulate synaptic strength, or synaptic plasticity, is the basis for normal nervous system functions such as learning, memory, movement, and perception. Synaptic plasticity occurs in two known forms, Hebbian learning and homeostatic plasticity, which have opposing effects on synaptic strength (Vitureira and Goda 2013; Zenke and Gerstner 2017). Hebbian learning consists of long-term potentiation (LTP) and long-term depression (LTD). In LTP, a synapse gains strength through increased activation that occurs in synchrony with the neuron’s firing (Bliss and Collingridge 1993; Malenka and Bear 2004). In LTD, the synapse loses strength through decreased activation and activation that occurs asynchronously with the neuron’s firing (Collingridge et al 2010; Malenka and Bear 2004). The resulting differences in synaptic strength are the basis for information storage in a neural network. However, both LTP and LTD are positive-feedback mechanisms due to the fact that stronger synapses are more likely to be activated: if left unchecked, LTP will lead to runaway excitation and LTD will lead to complete synaptic silencing. The second form of synaptic plasticity, homeostatic plasticity, responds to chronic changes in network activity in a negative-feedback manner by inducing opposing, network-wide changes in synaptic strength (O'Brien et al 1998; Turrigiano 2012; Turrigiano et al 1998). These changes maintain a network’s level of firing activity while preserving the relative synaptic strengths encoded by Hebbian plasticity.

**Homeostatic plasticity**

A pair of landmark studies published in 1998 (Turrigiano et al 1998; O'Brien et al 1998) presented evidence that synapses respond to chronic network silencing by globally strengthening synapses in a uniform multiplicative manner, which is accompanied by a return to previous levels of network activity (Figure 1). In Turrigiano et al, the authors recorded miniature excitatory post-synaptic currents (mEPSCs) in cultured rat cortical neurons after chronically inhibiting action potentials with the sodium channel blocker tetrodotoxin (TTX) and observed
that the amplitudes recorded in TTX-treated neurons were larger than those recorded in untreated control neurons. Conversely, neurons treated with bicuculine, a GABA receptor antagonist that increases firing activity by blocking inhibitory currents, showed smaller mEPSC amplitudes than in the control neurons. To determine the precise nature of the changes in mEPSC amplitude, the authors then devised a method to compare a distribution of mEPSC amplitudes recorded in treated neurons to a distribution of amplitudes recorded in untreated neurons and calculate the mathematical transformation between the two. Both mEPSC amplitude distributions were sorted from smallest to largest (rank-ordered), the untreated amplitudes were plotted against the treated amplitudes, and a linear regression model was fit to the data, yielding a slope and intercept coefficient that described the linear relationship of the treated data to the untreated data using the equation $y = mx + b$. The authors tested the accuracy of the coefficients by using them to mathematically scale the treated amplitudes. In both the TTX- and bicuculine-treated data, this produced a scaled distribution similar to that of the respective control amplitudes, indicating that the slope and intercept coefficients were an accurate mathematical approximation of the homeostatic effect and accurately reversed both an increase in amplitude resulting from decreased activity (the TTX data) and a decrease in amplitude resulting from increased activity (bicuculline). They concluded that homeostatic plasticity had a uniform multiplicative effect on mEPSC amplitudes and called the effect “synaptic scaling.”

**Involvement of glutamate receptor trafficking in homeostatic plasticity**

Although the full and precise mechanism of homeostatic plasticity remains unknown, the involvement of glutamate receptor trafficking in the homeostatic change in mEPSC amplitudes has been well documented. One of the landmark studies that established the synaptic scaling hypothesis also established that the changes in mEPSC amplitude resulting from both decreased
and increased activity were accompanied by corresponding changes in the level of postsynaptic AMPA receptors (O’Brien et al 1998). The homeostatic effect on receptor levels has been replicated in a number of subsequent studies (Wierenga et al 2005; Aoto et al 2008; Soden and Chen 2010; Scudder et al 2014; Fu et al 2011; Correa et al 2012; Shepherd et al 2006; Stellwagen and Malenka 2006; Koesters 2015), many of which have identified additional pathways and signaling molecules whose function is necessary to mediate the homeostatic effect on both receptor levels and mEPSC amplitudes. Most of these studies examine the effects of either decreased or increased activity, but not both; as a result, it remains an open question whether the homeostatic responses to increased and decreased activity are mediated by two different mechanisms or a single bidirectional mechanism.

Retinoic acid and one of its downstream targets, fragile X mental retardation protein (FMRP), are regulators of dendritic protein translation and promote the insertion of AMPA receptors at synaptic sites. Increased levels of retinoic acid have been shown to cause an increase in both synaptic strength and surface AMPA receptor expression, similar to the homeostatic increase that results from decreased firing activity. Decreased levels of retinoic acid abolish the homeostatic response to activity blockade, making it both necessary and sufficient for the homeostatic effect on mEPSC amplitude and surface receptors to be observed (Aoto et al 2008). The effects of retinoic acid were later shown to be mediated by its downstream target FMRP (Soden and Chen 2010), suggesting that disruption of the homeostatic plasticity mechanism may be responsible for at least some of the symptoms of fragile X syndrome.

Several molecules in the ubiquitination pathway, which regulates the degradation of proteins, have been shown to play a role in the homeostatic decrease in synaptic strength following an increase in firing activity (Scudder et al 2014; Fu et al 2011). Specifically, inhibiting ubiquitination
blocks the homeostatic decrease in both AMPA receptor levels and mEPSC amplitudes following treatment with bicuculline to increase network firing activity. The proteins that mediate this effect include the ubiquitin ligases Nedd4-1 and anaphase-promoting complex (APC), and the APC activator Cdh1.

The transcriptional repressor MeCP2, which blocks expression of the GluA2 AMPA receptor subunit, is one of the few proteins that has been shown to mediate the homeostatic response to both increased and decreased activity. Treatment with bicuculline, which increases neuronal firing activity, results in increased MeCP2 levels along with decreased AMPA receptors and mEPSC amplitudes, and downregulation of MeCP2 blocked the homeostatic decrease in both receptor levels and amplitudes (Qiu et al 2012). A separate study demonstrated the loss of MeCP2 also blocks the homeostatic increase in receptors and mEPSC amplitudes following activity blockade, both in vitro (AMPA receptor inhibition via DNQX) and in vivo (visual deprivation) (Blackman et al 2012), but did not explicitly show that activity blockade decreased the levels of MeCP2 either in vitro or in vivo. Interestingly, loss-of-function mutations in the MeCP2 gene are the predominant cause of Rett syndrome, which results in mental retardation. The above studies thus provide additional evidence that disruption of homeostatic plasticity may contribute to mental retardation.

A brain-derived neural factor (BDNF)-dependent pathway that includes MSK1 and Arc/Arg3.1 regulates surface AMPA receptor expression and has also been shown to play a role in both homeostatic up- and downscaling. Arc/Arg3.1 is necessary to produce an increase in mEPSC amplitude following activity blockade (Correa et al 2012; Shepherd et al 2006). Additionally, Arc/Arg3.1 has been shown to regulate homeostatic up- and downscaling of both receptor expression and mEPSC amplitude (Shepherd et al 2006).
While the majority of the pathways implicated in homeostatic plasticity are postsynaptic, the involvement of extracellular and presynaptic mechanisms in mediating the homeostatic response to decreased firing activity has also been observed. Stellwagen and Malenka (Stellwagen and Malenka 2006) demonstrated that the pro-inflammatory cytokine tumor-necrosis factor-α (TNF-α) is required for the homeostatic increase in mEPSC amplitude induced by activity blockade, and that the TNF-α responsible for mediating the increase was produced in the glia. The presynaptic vesicular fusion molecule Rab3A is also involved in mediating a homeostatic increase in mEPSC amplitude and receptor levels; no homeostatic effect is observed in neurons expressing Rab3A with a loss-of-function mutation, or in neurons in which Rab3A has been knocked out (Koesters 2015).

While most studies are in agreement that homeostatic plasticity involves a change in surface glutamate receptor levels, results regarding the involvement of specific receptor subunit types are less consistent. Some studies conclude that both the GluA1 and GluA2 subunits are equally or independently affected (Altimimi and Stellwagen 2013; Wierenga et al 2005), while others show that the homeostatic response is limited to or mediated by only one of the two subunits (Aoto et al 2008; Gainey et al 2009; Correa et al 2012; Garcia-Bereguaiain et al 2013). These differences have been attributed to experimental conditions such as species and tissue type.

**Limitations of the synaptic scaling hypothesis**

The previous section highlights the diversity of the signaling pathways and molecules involved in the mechanism of homeostatic plasticity. This complexity makes it essential to have a thorough and robust understanding of the mathematical transformation imposed on mEPSC amplitudes during a homeostatic response to altered firing activity, because the nature of this transformation dictates the types of pathways that should be considered in the search for the
homeostatic mechanism. For example, the current hypothesis is that homeostatic plasticity scales mEPSC amplitudes by a constant and global factor, which indicates that the mechanism responsible must modify each existing synapse in proportion to its initial strength, or to its initial number of functional receptors (Turrigiano et al 1998). The synaptic scaling hypothesis imposes the additional constraint that potential mechanisms must modify synapses simultaneously, as the homeostatic effect has been detected at multiple timepoints following the modification of firing activity. This constraint precludes the consideration of mechanisms such as (for example) antero- or retrograde dendritic transport, which would modify synapses based in part on their distance from the soma.

Following the introduction of the synaptic scaling hypothesis, subsequent homeostatic plasticity studies have found a similar increase in mEPSC amplitude across a wide variety of experimental conditions, in addition to the canonical preparation of dissociated cultures of rat cortical neurons treated with TTX. Synaptic scaling has been identified in many other in vitro experiments (Aoto et al 2008; Altimimi and Stellwagen 2013; Fong et al 2015; Fu et al 2011; Ibata et al 2008; Qiu et al 2012; Santin et al 2017; Scudder et al 2014; Shepherd et al 2006; Soden and Chen 2010; Stellwagen and Malenka 2006; Wierenga et al 2005) as well as in vivo (Desai et al 2002; Echegoyen et al 2007; Garcia-Berreguiain et al 2013; Goel and Lee 2007), with a number of studies performing experiments both in vitro and in vivo (Blackman et al 2012; Cingolani and Goda 2008; Correa et al 2012; Gainey et al 2009). In addition to studies using rat neurons, homeostatic plasticity studies have identified synaptic scaling in the mouse (Altimimi and Stellwagen 2013; Blackman et al 2012; Goel and Lee 2007; Soden and Chen 2010; Teichert et al 2017) and the bullfrog (Santin et al 2017). Different tissue types include the hippocampus (Altimimi and Stellwagen 2013; Echegoyen et al 2007; Qiu et al 2012; Scudder et al 2014; Shepherd et al 2006; Soden and Chen 2010), the visual and auditory cortices (Blackman et al 2012; Cingolani and Goda 2008; Correa et al 2012;
2012; Desai et al 2002; Gainey et al 2009; Goel and Lee 2007; Teichert et al 2017), respiratory motoneurons (Santin et al 2017), and the spinal cord (Garcia-Bereguiain et al 2013; O'Brien et al 1998). The pharmacological methods for modifying firing activity to elicit a homeostatic response include action potential inhibition via TTX, glutamate receptor inhibition via antagonists such as CNQX or DNQX, and reducing inhibition via bicuculline, a GABA inhibitor. Studies in the sensory cortices often use sensory deprivation to reduce input to the cortex and produce a homeostatic increase in synaptic strength, and hibernation has been shown to induce homeostatic plasticity in the respiratory neurons.

Although many of these studies were consistent with the hypothesis of synaptic scaling (Desai et al 2002; Garcia-Bereguiain et al 2013; Ibata et al 2008; Santin et al 2017; Teichert et al 2017; Turrigiano et al 1998), some studies have found that multiplicative scaling did not recapitulate the homeostatic effect in their data (Echegoyen et al 2007; Cingolani and Goda 2008; Goel and Lee 2007). Additionally, a number of the studies that do claim scaling do so without providing any quantitative analysis (Craig and Henley 2012; Martin et al 2015; Stellwagen and Malenka 2006) and/or in spite of noticeable deviations of their scaled data from control (Ibata et al 2008). A recent review acknowledged the presence of homeostatic mechanisms that are not global and do not strictly multiplicatively scale, but continued to invoke synaptic scaling as an important plasticity mechanism (Turrigiano 2012). Although the presence or absence of uniform synaptic scaling could be a function of species or tissue type, the wide variety of experimental conditions under which homeostatic plasticity has been studied means that the exact experimental conditions are seldom duplicated, making it difficult to draw confident conclusions. The possibility that homeostatic plasticity does not always produce uniform synaptic scaling is investigated in Specific Aims 1 and 3.
It should also be noted that the rank-order method of computing the synaptic scaling equation uses the intercept coefficient as well as the slope coefficient of the linear fit in the mathematical transformation meant to reverse the homeostatic plasticity effect. Because the intercept coefficient is an additive factor, its inclusion results in a transformation that is not purely multiplicative. Although it has been suggested that the intercept coefficient corrects for the experimental limit on the detection of mEPSC amplitudes (Blackman et al. 2012), no evidence in support of this view has been provided. Recently, Kim and colleagues attempted to address the concern that the transformation computed by the rank-order method is not uniformly multiplicative, and proposed a new method to correct for any detection threshold without the use of an additive factor (Kim et al. 2012). They concluded that their method was successful; however, their approach was based on an arbitrarily-chosen significance threshold, without which their conclusion may not have been supported. An improved method that addresses and corrects this statistical shortcoming is proposed in Specific Aim 2.

A large part of the value of the synaptic scaling hypothesis is that it provides a set of constraints on the possible mechanism of homeostatic plasticity. As discussed above, such constraints are essential to direct and limit possible avenues of research. For this reason, a more rigorous investigation into the validity and applicability of the synaptic scaling hypothesis is necessary. However, any new hypothesis must also provide explanations for how the relative synaptic weights developed through Hebbian learning are maintained, and how runaway excitation and inhibition are prevented.
II. METHODS

**Electrophysiology**

The first data set used in this study was previously recorded in our laboratory: homeostatic plasticity was induced in 13-14 days in vitro (DIV) dissociated cultures of mouse cortical pyramidal neurons by inhibiting action potentials with 500 nM TTX for 48 hours. Miniature excitatory post-synaptic currents (mEPSCs) were then recorded from visually-identified pyramidal-shaped neurons via voltage clamp, and these recordings were analyzed to obtain the mEPSC amplitudes.

Three additional, previously published data sets were obtained from our collaborators. Two of these were recorded in dissociated cultures of rat cortical pyramidal neurons (Fong *et al* 2015); one experiment used TTX to induce homeostatic plasticity and the other used CNQX, a glutamate receptor inhibitor. The third data set was recorded in dissociated cultures of mouse hippocampal neurons, which were also treated with TTX (Altimimi and Stellwagen 2013). The experimental conditions for all four data sets are summarized in Table 1.

**Sampling**

Because the number of mEPSCs recorded varies by cell, the data must be sampled to ensure that each cell contributes to the data a representative subset of equal size. Initially, a random subset of events with $n = 30$ was chosen without replacement from each cell; the events from untreated neurons were pooled to form the CON distribution, and events from TTX-treated neurons were pooled to form the TTX distribution. A sampling approach based on random
selection has the potential for sampling error, the generation of a non-representative sample due to random chance. To eliminate this possibility, we also used a quantile-based approach. Quantiles are values that describe a frequency distribution by dividing it into equal groups, such that each value represents the same fraction of the total data. For example, the median divides a distribution into two halves and represents the midpoint, or 50th percentile. We performed quantile sampling by computing 30 evenly-spaced quantile values from every cell, starting at the 1.67th percentile (one-half of 1/30) and ending at the 98.33th percentile (100% minus one-half of 1/30) with a step size of 3.33% (1/30). These particular sampling values were chosen to avoid including the cells’ absolute minimum or maximum mEPSC amplitude in the sample, as the amplitudes at either extreme tended to be sparse and widely variable. As before, the quantiles from untreated neurons were pooled to form the CON distribution, and the quantiles from TTX-treated neurons were pooled to form the TTX distribution.

**Empirical simulation of homeostatic plasticity**

We created an empirical simulation of homeostatic plasticity based on the prevailing hypothesis of uniform multiplicative synaptic scaling. Two distributions of events, simCON1 and simCON2, were generated by choosing 30 random mEPSC amplitudes from each control neuron. One of these distributions (simCON2) was multiplied by a scaling factor of 1.25 to simulate the hypothesized effect of treating neurons with TTX, generating distribution simTTX. The other distribution (simCON) was not manipulated and was used as the untreated control data. Two different experimental detection thresholds were also simulated by truncating both distributions, after multiplication, at a low threshold (5 pA) and a high threshold (7 pA): all values below the threshold were discarded from the distributions to approximate the way an experimental detection threshold prevents small events from being recorded. Because truncation removed a greater number of events from the simCON distribution for both
threshold values, events were then randomly discarded from the simTTX distributions so that simCON and simTTX had the same sample size across all simulations. Unlike the experimental data, the same-sized simCON and simTTX distributions were used for both the ranked and the cumulative plots, as opposed to only the ranked plot, for ease of use. The results of the simulation are shown in Figure 5.

**Rank-order test for uniform multiplicative scaling**

The rank-order method (Turrigiano et al 1998) is the most widely-used test for uniform multiplicative scaling in homeostatic plasticity data. Because this method requires the data to be fit with a linear regression model, the data must be resampled to obtain an equal number of samples in both the treated and untreated distribution. Previous studies have accomplished this by randomly discarding events from the larger distribution; although we used this approach to obtain distributions of equal size from our simulation, we chose to use quantile sampling on all experimental data to avoid the potential for sampling error. In order to obtain pooled distributions of the same size, 77 quantiles were sampled from control cells (N = 87) and 87 quantiles were sampled from the TTX-treated cells (N = 77). Thus both pooled distributions had a sample size of 6699 (77 quantiles * 87 cells for control; 87 quantiles * 77 cells for TTX). The equal-size amplitude distributions were then ranked and fitted with a linear regression model \( y \sim mx + b \) using control as the predictor (x) and TTX as the response (y) to obtain the slope (m) and intercept (b) coefficients. The original TTX distribution (with 30 quantiles/cell) was then downscaled by the coefficients of the regression model using the equation \((\text{TTX} – b)/m\), and the downscaled TTX data were compared to the original CON distribution with either a Kolmogorov-Smirnov or Anderson-Darling test for the equivalence of distributions (see “Statistical analysis” section below for details). A statistically significant difference (\(p < 0.05\)) between the downscaled TTX data and the control data indicates that the method has failed to find uniform
multiplicative scaling in the data, and the lack of significant difference is considered to be a success and an indication that the data are uniformly scaled.

**Iterative test for uniform multiplicative scaling**

The iterative process to determine the scaling factor was proposed by Kim and colleagues (Kim et al. 2012) as an alternative to the rank-order method for testing whether homeostatic plasticity results in uniform multiplicative scaling. This method repeatedly downscales the pooled TTX data by an arbitrary factor, discards any downscaled amplitudes smaller than the smallest amplitude in the control data, and compares the fit of the downscaled TTX data to the control data with a Kolmogorov-Smirnoff test for equivalent distributions. Because the goal of this process is to find the transformation that results in the best fit between the downscaled TTX and control data, large p-values indicate success (minor differences between the distributions) and small p-values indicate failure (significant differences between the distributions). The process is repeated for scaling factors across a range of values, and the factor that produces the largest p-value, indicating the best fit between the downscaled TTX and control data, is chosen. We applied the process both as described and without the removal of events that fell below the detection threshold.

**Statistical analysis**

The Kolmogorov-Smirnov test for the equality of distributions has previously been used to analyze the similarity of two groups of mEPSC amplitudes. Its test statistic is a function of the point of greatest deviation between the distributions being compared, and it tends to be more sensitive to deviations near the middle (median) of the distributions. Another test for the equality of distributions, the Anderson-Darling test, has a test statistic that is a function of the absolute value of the area between the distributions being compared, and is more sensitive to
deviations in the distributions’ tails (Scholz and Stephens 1987). Because we are more interested in
the relative shape of the mEPSC amplitude distributions than whether they share a similar
median, and because we believe slight but consistent deviation should be considered
meaningful, we believe that the Anderson-Darling test is better suited for use on this type of
data, although we continue to use the Kolmogorov-Smirnov test in the iterative test and several
other cases for consistency with previous studies.

However, because multiple amplitudes are recorded per cell, the data in the mEPSC amplitude
distributions are not independent and identically distributed (iid), which is a requirement of
both the K-S test and the A-D test. Violation of a statistical test’s requirements means that the
distribution of the test statistic, and thus the critical value, is unknown, and the test cannot be
used to draw conclusions regarding statistical significance. Furthermore, the power of both tests
increases with sample size, meaning that larger samples will, on average, yield smaller p-values
than smaller samples. This has the potential to make it more likely to find an accurate
transformation in small data sets than in large data sets, because the larger data sets will tend
to produce smaller p-values due to the increased sensitivity of the test to minor deviations. For
these reasons, we use these tests only to compare the fit between pairs of similarly-sized
distributions, and not to judge whether two distributions are significantly different or to
compare fits between data sets with different sample size.

In the cases where statistical significance is desired, a critical value can be empirically
determined using a test statistic distribution obtained by repeatedly applying the test to mEPSC
amplitude data (see next section for details). For any level of statistical significance $\alpha$, the critical
value is the $(1 - \alpha)$th percentile of the test statistic distribution, e.g. if $\alpha = 0.05$, the critical value
is the 95th percentile of the distribution. Similarly, a p-value for a specific instance of the test can
also be computed. A p-value indicates the probability under the null hypothesis of obtaining
greater deviation between the distributions than what is observed; therefore, a p-value is
calculated as the fraction of the test statistic distribution greater than the observed test statistic.

**Bootstrap estimation of parameters**

Monte Carlo case resampling is a type of bootstrapping procedure commonly used to obtain an
estimate of the sampling distribution of a population statistic such as the mean or median by
resampling data from that population. Simulated samples are generated by sampling with
replacement from the original dataset; the simulated sample must be the same size as the
original data. The statistic of interest is computed on the simulated sample and saved. These
steps are repeated many times, resulting in a bootstrap distribution of the statistic of interest.
Because the simulated samples are sampled with replacement, some of the values from the
original sample may appear in a simulated sample multiple times, while others do not appear at
all. This creates variance among the simulated samples that causes the variance in the bootstrap
distribution. The bootstrapping procedure thus approximates the results that would be obtained
by repeatedly sampling data from the population.

Here, we used bootstrapping to generate bootstrap distributions of the slope and intercept
coefficients produced by the rank-order test for synaptic scaling. A flow diagram of the
bootstrapping procedure is shown in Figure 2. The simulated samples for the bootstrapping
procedure consisted of two groups of neurons with N = 87 sampled randomly with replacement
from the untreated neurons in our experimental data. For each group, the neurons were
quantile sampled and the sample values were pooled and sorted to produce an amplitude
distribution. The rank-order method was used to compute the coefficients for the
transformation function to fit the Group 1 data to the Group 2 data, and then the
transformation function was applied to the values in the Group 1 distribution to produce the Scaled distribution. The fit of the Scaled distribution to the Group 2 distribution was evaluated using an Anderson-Darling test, and the test statistic from this test was saved, along with the factors of the transformation function. The output from the procedure consisted of bootstrap distributions of the slope and intercept coefficients, and of the test statistic. The bootstrap distributions of the coefficients were used to assess the accuracy and precision of the model by evaluating the distributions’ location and width, respectively. The bootstrap distribution of test statistics was used to determine the correct critical value for a given level of statistical significance, and to compute the p-value of specific instances of the transformation.
III. SPECIFIC AIM 1

Demonstrate the insufficiency of uniform multiplicative scaling as a model of homeostatic synaptic plasticity in cultured mouse cortical neurons.

Rationale

Uniform synaptic scaling is so widely accepted that in some cases, studies use the term based solely on the appearance of the control and treatment distributions of mEPSC amplitude and either the scaled fit or the results of a statistical test are not shown (Teichert et al. 2017; Stellwagen and Malenka 2006; Martin et al. 2015; Craig and Henley 2012; Shepherd et al. 2006; Soden and Chen 2010). As a result, data sets that are considered uniformly scaled can appear quite dissimilar from one another, which calls into question whether they really all exhibit the same trend.

The original study establishing synaptic scaling included an additive term in the equation used for the scaling transformation (Turrigiano et al., 1998). The requirement for an additive term suggests that homeostatic plasticity cannot be described by a multiplicative factor alone; although it has been stated that the intercept compensates for the experimental detection threshold (Blackman et al. 2012), no rationale or supporting evidence for this statement has been published. Kim and colleagues attempted to address whether the intercept is a consequence of a detection limit by using an iterative process to test potential scaling factors (Kim et al. 2012). The iterative process with threshold correction was able to scale the data with a multiplicative factor alone, as determined by a K-S test, and the authors concluded that
homeostatic plasticity is indeed simply multiplicative, but the statistical methods used to reach this conclusion were opaque and questionable. Additionally, a recent review acknowledges the presence of homeostatic mechanisms that are not global and do not strictly multiplicatively scale, although the review continues to invoke synaptic scaling as an important plasticity mechanism (Turrigiano, 2012).

A large homeostatic plasticity data set previously collected in our laboratory has a relatively small experimental detection threshold (3 pA) but exhibits a marked overlap between the smallest control and TTX-treated mEPSC amplitudes, which precludes uniform multiplicative scaling. Similar overlaps have been observed in previous studies (Blackman et al 2012; Correa et al 2012; Echegoyen et al 2007; Ibata et al 2008). We hypothesize that our homeostatic plasticity data exhibit non-uniform scaling.

**Approach**

The hypothesis that the uniform scaling model of homeostatic plasticity is insufficient to explain the trends observed in our data will be tested using an empirical simulation approach. We will create a simulation of uniformly multiplicative scaling which will allow us to observe the expected appearance of data that support the uniform scaling hypothesis. Characteristics such as high variability and truncation by an experimental detection threshold will also be simulated to determine whether these can cause uniformly-scaled data to appear non-uniform. Our hypothesis is supported if the data resulting from these simulations are dissimilar to the experimental data, as this indicates that uniformly-scaled data cannot be made to appear non-uniform by these characteristics and the non-uniformity is likely to be a true aspect of the data, not an artifact.
The rank-order and iterative methods will then be performed on the simulation data to observe the expected outcome of these tests on uniformly-scaled data with and without a detection threshold and determine whether a detection threshold can prevent the tests from finding uniform scaling. The results of the rank-order and iterative tests on our experimental data will then be compared to the results on the simulation data. If a detection threshold does not prevent the rank-order and/or iterative methods from detecting uniform scaling, but the methods fail to find uniform scaling in the experimental data, our hypothesis is supported.

Finally, we will use a novel plot of the ratio of TTX to CON amplitudes to visualize the plasticity effect as a function of the control amplitudes in both the simulation and the experimental data. We expect that the ratio will be roughly constant in uniformly-scaled data; the ratio plots of the simulation data will verify this expected result and demonstrate the effect of truncation on the ratio. Our hypothesis is supported if the ratio plot of our data is not constant and does not resemble the ratio plots of truncated data.

**Results**

Experiments to induce and characterize homeostatic plasticity were previously performed in our laboratory (Koesters 2015): dissociated 13-14 DIV cultures of mouse cortical neurons were treated with 500 nM tetrodotoxin (TTX) for 48 hours to block firing activity, and the amplitudes of miniature excitatory post-synaptic currents (mEPSCs) recorded in neurons from these cultures were compared to the mEPSC amplitudes recorded in untreated control neurons. Figure 3A shows a typical pyramidal-shaped neuron that was chosen for recording. Individual current traces recorded from an untreated and a TTX-treated cell are shown in Figure 3B, and the average mEPSCs waveforms from these recordings are shown in Figure 3C. The mean mEPSC
amplitudes from control and TTX-treated cells were significantly different (CON, 13.9 ± 0.4 pA; TTX, 17.5 pA ± 0.5 pA; p = 4.92 x 10^{-7}, Kruskal-Wallis test; Figure 3D).

**Experimental data do not resemble a simulation of uniform scaling**

Homeostatic plasticity is typically analyzed by comparing the cumulative distribution functions (CDFs) of mEPSC amplitudes from control and TTX-treated cells. We generated CDFs of mEPSC amplitudes by randomly sampling 30 events per cell and pooling these samples for each experimental condition (Figure 4A). To compare these plots to the expected appearance of uniformly scaled data, we simulated perfectly uniform, multiplicative scaling by multiplying the distribution of control events (CON) by 1.25 and plotting it alongside the original CON distribution (Figure 4B). The CDF derived from uniform scaling differs from published studies as well as the plots of our experimental data: uniformly scaled data shows separation from CON data throughout, whereas experimental TTX distributions commonly overlap closely with CON in the smallest amplitudes, with the separation widening as amplitudes increase.

**The appearance of non-uniformity is not due to sampling error or mEPSC variability**

We were concerned that the apparent deviation of our data from uniform scaling could be a byproduct of sampling error, which is defined as obtaining a non-representative sample due to random chance. We resampled our data by computing 30 evenly-spaced quantiles from each cell’s mEPSC distribution (see Materials and Methods for details). The quantile values were used in place of the 30 random mEPSC amplitudes to form the pooled CON and TTX distributions, thus removing the random element from the sampling process and ensuring a representative sample. Aside from smoothing the pooled distributions, quantile sampling did not alter the appearance of the experimental data (Figure 4C).
Biological data is by its nature highly variable, and the variability in mEPSC amplitudes is well documented (Bekkers et al 1990; Liu and Tsien 1995; McAllister and Stevens 2000). We used an empirical simulation approach to investigate whether high variability could cause uniformly scaled data to appear non-uniform. To perform the empirical simulation, we randomly sampled 30 mEPSC amplitudes from every untreated cell to create simulated control sample 1 (simCON1), then randomly sampled again to generate simulated control sample 2 (simCON2). To obtain two data sets that were different due to the variability between mEPSCs, we repeated the sampling process until a Kolmogorov-Smirnov (K-S) test for the equality of distributions yielded a small p-value (p = 0.04; Figure 5A, solid black line and dashed vermillion line). The second resampled control distribution, simCON2, was then multiplied by 1.25 to produce the simulated TTX distribution, simTTX (Figure 5A, vermillion solid line). Because of the variation between simCON1 and simCON2, the actual scaling factor between simCON1 (subsequently referred to as simply simCON) and simTTX is 1.31 instead of 1.25; that is, simCON2 was roughly 1.06 times simCON1. In spite of the variability, simCON and simTTX retained the appearance of uniform scaling, with the simTTX CDF separated from the simCON CDF throughout (Figure 5A, solid black line and solid vermillion line). This result suggests that high variability cannot cause a uniformly-scaled data set to appear non-uniformly-scaled, and therefore that the non-uniform appearance of our experimental data is not a consequence of mEPSC variability.

A high detection threshold can cause uniformly-scaled data to appear slightly non-uniform

It has been suggested previously that an experimental detection threshold could cause mEPSC amplitude distributions to deviate from uniform, multiplicative scaling (Blackman et al 2012; Kim et al 2012). By failing to detect the smallest mEPSCs, a detection threshold removes a higher proportion of events from untreated control neurons than from TTX-treated neurons, resulting in an uneven truncation of the data which creates a mismatch between the two distributions. To
test whether a detection threshold could cause uniformly scaled data to appear non-uniform, we used the data from the simulation of uniform scaling shown in Figure 5A to simulate a detection threshold of 5 pA by discarding all mEPSC amplitudes below this threshold from both the simCON and simTTX distributions (Figure 5B). A higher threshold of 7 pA was simulated in the same manner (Figure 5C). The thresholds are shown relative to the original distributions on an expanded scale (Figure 5A-C, insets); both threshold values eliminate a greater proportion of events from simCON than simTTX, although fewer events are removed by the 5 pA threshold. Simulation of a 7 pA detection threshold produced data in which there is a slight overlap in the smallest amplitudes in simCON and simTTX; the overlap is present to a lesser degree in the data from the simulation of a 5 pA threshold (Figure 5B-C). Although neither simulated detection threshold exactly reproduced the extent of the overlap seen in the experimental data, these results suggest that a high detection threshold could potentially cause uniformly-scaled data to appear slightly non-uniform on a cumulative distribution plot. It is thus possible that the apparent non-uniformity in our experimental data could be due to a detection threshold, but the threshold in our data is small (3 pA) compared to the simulated threshold that produced non-uniformity (7 pA).

The rank-order method does not find uniform scaling in experimental data

The rank-order method is a commonly used tool for analyzing homeostatic plasticity data. In this process, the CON and TTX mEPSC data are ranked from the smallest to largest amplitude, plotted against each other, and fit with a linear regression model to yield a slope and an intercept coefficient. These coefficients are used to quantify the mathematical transformation induced by homeostatic plasticity on the mEPSC amplitudes in TTX-treated neurons. If the coefficients accurately describe the effects of homeostatic plasticity, then using them to mathematically downscale the TTX distribution should reverse those effects and produce a
scaled distribution similar to the CON distribution. Note that the inclusion of the additive intercept coefficient results in a transformation that is not uniformly multiplicative; the use of the intercept term is believed to correct for any misalignment of the ranked data caused by a detection threshold (Blackman et al 2012). We wished to test this assumption by demonstrating the rank order method’s results on data from our empirical simulations of uniform scaling, both with and without a detection threshold. If the method calculates a transformation that produces a good match between the scaled TTX and control distributions even in the presence of a high detection threshold, then it should also be capable of correcting for any detection threshold in our experimental data and calculating a good match there as well, assuming that the experimental data are truly uniformly scaled.

We applied the rank order method to data from our simulation of uniform scaling and found that, as expected, it produced a scaled simTTX distribution with an excellent fit to simCON ($N_{\text{simCON}} = 2610, N_{\text{simTTX}} = 2610, p = 0.769$; Figure 6A-B). The slope coefficient was close to the expected value (slope = 1.316, expected value 1.31) and the intercept coefficient was -0.27, close to the expected value of 0 for untruncated data. In the low-threshold simulation data, the process also resulted in a very good fit between the scaled simTTX and simCON distributions ($N_{\text{simCON}} = 2594, N_{\text{simTTX}} = 2594, p = 0.628$; Figure 6C-D), with a slope coefficient close to the expected value of 1.31 (slope = 1.32) and an intercept coefficient of -0.37. The marginal increase in magnitude of the intercept from the intercept of the untruncated simulation data is consistent with the observation that the simulated 5 pA detection threshold removed only a small amount of data from the distributions, requiring only a small correction. In the high-threshold data, the process produced a scaled distribution that matched well with the upper quantiles (>75%) of simCON but was misaligned in the lower quartile ($N_{\text{simCON}} = 2321, N_{\text{simTTX}} = 2321, p = 0.012$; Figure 6E-F, see inset in E). Although the slope coefficient remained unchanged,
indicating that the accuracy of the method was unaffected by the high threshold, the intercept coefficient was only -1.24, a surprisingly small value given the high magnitude of the 7 pA detection threshold and the fact that the intercept coefficients observed in the literature are typically -2 to -6, with larger magnitudes occasionally observed (Altimimi and Stellwagen 2013; Fong et al 2015; Echegoyen et al 2007; Turrigiano et al 1998). In both detection threshold simulations, the smallest amplitudes in the ranked data have a much steeper slope than the majority of the data, creating the appearance of a droop (Figure 6D, F), which serves as a useful visual indicator of the detection threshold. Overall, these results show that, on uniformly scaled data, the rank-order method was capable of calculating a nearly perfect match between the simulated control and scaled TTX distributions with no or a small detection threshold. A large detection threshold had a moderate adverse effect on the match but did not impact the method’s ability to find the correct slope coefficient. The magnitude of the intercept coefficient did increase with the value of the detection threshold, although not to the expected extent. We thus expect that, if our data are uniformly scaled, the rank-order method will produce an excellent match between the scaled TTX and control distributions, given that our detection threshold was small (3 pA). However, the rank-order method on the experimental data produced a scaled TTX distribution that was a very poor fit to the CON distribution \(N_{\text{CON}} = 2580, N_{\text{TTX}} = 2310, p = 6.0 \times 10^{-10}; \) Figure 7A), and the ranked data did not exhibit the droop (Figure 7B). The deviation of this outcome from the expected results of the rank-order method on uniform-multiplicative scaled data therefore suggests that our data do not exhibit uniform multiplicative scaling.

**The iterative method does not find uniform scaling in experimental data**

The possibility that a detection threshold could disrupt the detection of uniform scaling has also been addressed by Kim and colleagues (Kim et al 2012), who developed an approach distinct
from that of the rank-order process. Their approach used a series of multiplicative factors to
downscale the mEPSC amplitudes from TTX-treated cells, and any downscaled amplitudes that
fell below the detection threshold (defined as the smallest observed mEPSC amplitude in the
control data) were discarded, thereby correcting the mismatch caused by the threshold. The fit
of the downscaled TTX mEPSC distribution to the CON distribution was then evaluated with a
Kolmogorov-Smirnov test; the scaling factor that produced the closest match between the
distributions, which corresponds to the largest p-value, was chosen. If the chosen scaling factor
produces a good fit between the downscaled TTX and control amplitudes, this supports
uniform multiplicative scaling in those data. To verify the validity of this approach, we applied
this process to the high-threshold simulation data and found that it almost completely
eliminated the effects of the truncation, producing an almost perfect fit between the scaled TTX
and CON distributions (\(N_{\text{simCON}} = 2321, N_{\text{simTTX}} = 1997, p = 0.97\); Figure 8A-B). To verify that the
goodness of the fit was a function of the detection threshold correction and not the
optimization of the scaling factor, we repeated the process without discarding sub-threshold
events from the downscaled TTX data. This version of the process produced a markedly worse fit
(\(N_{\text{simCON}} = 2321, N_{\text{simTTX}} = 2321, p = 0.00238\); Figure 8C-D), confirming both that the threshold was
responsible for the poor fit, and that discarding subthreshold events from the downscaled
distribution effectively corrected for it. However, applying the iterative process (including the
threshold correction) to the quantile-sampled experimental data produced a downscaled TTX
distribution that was a poor fit to the CON distribution (\(N_{\text{CON}} = 2580, N_{\text{TTX}} = 2308, p = 3 \times 10^{-5}\);
Figure 9), a result that fails to support the presence of uniform multiplicative scaling in our data.
Given that the fit was not substantially affected by leaving out the correction for the detection
threshold (Figure 9A, open circles vs. crosses), we also conclude that our data were probably not
 truncated by our experimental threshold of 3 pA.
The rank-order method with a valid test for statistical significance does not find uniform multiplicative scaling in experimental data

Although the Kolmogorov-Smirnov test is widely used to compare distributions of mEPSC amplitudes, there are several issues with this usage. The first issue is that the power of the K-S test, like most statistical tests, increases with sample size. Because the sample size of many homeostatic plasticity data sets is relatively high (1000’s of samples per group), the test has an extremely high power and will return small p-values when comparing large distributions that have only minor deviations from one another. The result is that the K-S test will reject the null hypothesis even when the small magnitude of the deviation between the distributions is unlikely to have functional biological implications. Exacerbating this tendency is the fact that the null hypothesis of the K-S test states that the sample distributions being compared originated from the same parent distribution; in the specific case of homeostatic plasticity data, this translates to the hypothesis that the two sets of mEPSC amplitudes were generated in the same cells. The mEPSCs in homeostatic plasticity experiments are typically recorded from sister cultures in order to maximize the similarity between the cells in the experimental groups, but the fact remains that the cells are not identical – some variation between the groups is inevitable, and this will also cause the test to return smaller p-values than if the groups were the same. The final and most serious issue is that the K-S test requires that the samples in each group be independent and identically distributed (i.i.d), a requirement that is violated by the use of multiple mEPSCs recorded in the same cell. The requirements of a statistical test define the conditions under which the distribution of the test statistic is known. Because both the critical value of a statistical test and its p-values are calculated using that distribution, violating the requirements of a test renders any conclusions regarding statistical significance invalid.
Because the failure of the rank-order and iterative methods to find uniform multiplicative scaling in our experimental data is both unexpected under current hypotheses and partially based on a flawed statistical test, we performed a bootstrap procedure to address the violation of the Kolmogorov-Smirnov test’s requirements. Bootstrapping uses repeated random sampling of an observed sample to estimate the parameters of the population from which the sample was drawn. Our procedure repeatedly resampled two distributions of events from our untreated control cells, used the rank-order method to find the mathematical transformation between the first distribution and the second, then performed a K-S test comparing the scaled second distribution to the unscaled first one. This process was repeated 10,000 times, resulting in bootstrap distributions of the slope coefficient, the intercept coefficient, and the K-S test statistic (Figure 2). On average, there should be no difference between two groups of mEPSC amplitudes sampled from the same set of control cells aside from the variability that arises through random sampling, so the bootstrap distributions of the coefficients can be used to assess the accuracy and precision of the rank-order method. The bootstrap distribution of the slope coefficient has a mean of 1 and the bootstrap distribution of the intercept coefficient has a mean of 0 (Figure 10A-B); these coefficients correspond to a transformation by identity, which is the expected outcome when using the rank-order method to compare two distributions of mEPSC amplitudes from the same population of cells. The bootstrap distribution of the K-S test statistic was used to obtain a statistically valid critical value by computing the 95th percentile (for a significance level of 0.05) of the distribution, resulting in a critical value of 8.37 (Figure 10C). However, the rank order method on our experimental data produced a scaled TTX distribution that, when compared to control, yielded a K-S test statistic of 29.6 (Figure 10D), a value that exceeds the critical value and causes the null hypothesis to be rejected, upholding the conclusion that uniform scaling is not observed in our data.
The ratio of TTX to CON amplitudes is uniform in simulations of uniform multiplicative scaling, but not in experimental data.

Thus far, our findings show that the deviation of the experimental data from the behavior expected for uniform multiplicative scaling cannot be attributed to sampling error, high variability, or a detection threshold, and that the deviation is statistically significant. In order to more directly observe how the magnitude of the homeostatically-induced scaling factor deviated from a single uniform value across mEPSC amplitudes, we plotted the ranked mEPSC data as the ratio of TTX/CON against CON for each pair of ranked amplitudes. In uniformly scaled data, the ratio should be roughly constant across amplitudes. A detection threshold should manifest as a reduced ratio at the smallest amplitudes, and the extent of the threshold’s effect on the data will be visible as the magnitude and range of this reduction. We plotted the data from the simulation of uniform scaling with no detection threshold and confirmed our expectation that the ratio plot would yield a constant value across amplitudes (Figure 11A). The ratio plot of the simulation data with a 5 pA threshold also exhibited a constant value over most of the mEPSC amplitudes and, also as expected, the ratio was underestimated for approximately the smallest 10% of the data (Figure 11B). For data with the larger (7 pA) threshold, the ratio was underestimated for a greater proportion of the data, roughly the bottom quartile, after which the value plateaued near the expected value (Figure 11C). In each of these cases, the plateau approaches the expected scaling factor of 1.31 (dashed vermillion lines), which is the combination of the simulated scaling factor of 1.25 and the scaling factor of 1.06 caused by variability between the simulated samples. In dramatic contrast, the ratio calculated for the experimental data increased gradually over more than 60% of the data before reaching an approximate plateau (Figure 11D). Not only is this proportion of the data far greater than can be attributed to a detection threshold, the ratio does not reach its plateau anywhere near the slope
factor determined from the linear regression fit. Taken together, these results clearly demonstrate that the scaling induced in our data by homeostatic plasticity is not uniform. Since the scaling factor is smallest for small amplitude mEPSCs, and increases with increasing amplitude, we propose a new name for this type of homeostatic transformation, “divergent scaling,” to distinguish it from uniform, multiplicative scaling.

Discussion

We have used empirical simulations to demonstrate the expected appearance of data that exhibit uniform multiplicative scaling, and shown that neither sampling error, high variability, nor truncation by an experimental detection threshold can cause uniformly scaled data to appear non-uniform. The simulation data bore an unexpected lack of resemblance to both our experimental data and other published homeostatic plasticity data: where the experimental data regularly exhibit overlap between control and treated mEPSC amplitude distributions in the smallest amplitudes, the simulations show that uniformly-scaled data should exhibit very little overlap in cases where the data are truncated by a high (> 5 pA) detection threshold, and none at all in the case of lower thresholds. These observations support our hypothesis that our data are not uniformly scaled and suggest that many published data sets may also exhibit non-uniformity, a possibility that will be further investigated in Specific Aim 3.

We then demonstrated that two established tests for uniform multiplicative scaling, the rank-order and iterative methods, were able to find the expected transformation in the uniformly-scaled simulation data even when the data were truncated by a high detection threshold. Surprisingly, given the widespread belief that the rank-order method corrects for truncation with an increased intercept coefficient, the intercept coefficients for all transformations were quite small (between 0 and -2). The transformations computed by both the rank-order and the
iterative methods also produced a nearly perfect visual match between the control and scaled simulation data sets for all values of the simulated detection threshold. While previous applications of these methods to experimental data have usually produced good fits, there are typically some minor deviations visible by eye; the goodness-of-fit of the transformations on the simulation data was unexpectedly high by comparison. This result indicates that both the rank-order and the iterative method are capable of very accurately computing the scaling transformation in data that exhibit uniform multiplicative scaling, and that truncation of the data does not cause a substantial decrease in the accuracy of the computed transformation, or in the quality of the match between the control and scaled distributions. However, neither the rank-order method or the iterative method computed a transformation that resulted in a close match in our experimental data. The use of a test statistic distribution obtained through a bootstrapping procedure confirmed that the scaled distribution produced by the rank-order test was significantly different from the control distribution, objective evidence that the mathematical transformation computed by the rank-order method did not accurately recapitulate the transformation between TTX-treated and untreated mEPSC amplitudes in mouse cortical neurons. Because we have shown that both methods are capable of finding a uniform multiplicative scaling transformation when one is present in the data, we believe that the failure of the methods to find a good match in mouse cortical neurons is an indication that these data do not exhibit uniform multiplicative scaling, a finding that supports our hypothesis.

To directly visualize the transformation between control and TTX-treated mEPSC amplitudes, we used a novel plot of the ratio of TTX to CON amplitudes. As expected, the ratio was roughly constant in the uniformly-scaled simulation data, with minor downward deviations in data that had been truncated by a simulated experimental detection threshold. These deviations caused the ratio to be underestimated in approximately 10% of the data in the case of the lower
detection threshold (5 pA), and 25% of the data in the case of the higher detection threshold (7 pA). In stark contrast to the simulation data, the ratio of TTX to CON in the experimental data increased steadily over nearly 70% of the data. The extent of the non-uniformity was far greater than that which can be attributed to a detection threshold. Our hypothesis that our data set does not exhibit uniform multiplicative scaling has thus been supported by every result in this specific aim.

In addition to answering the question of “uniform vs. non-uniform,” the ratio plot also provides a qualitative view of how the homeostatic scaling transformation affects mEPSCs of different amplitudes. The ratio of TTX to CON in our experimental data is small – barely greater than one – in the smallest amplitudes and increases with larger amplitudes. This observation is consistent with our previous observation that our TTX and control mEPSC amplitude distributions overlap in the smallest amplitudes and then separate. Because this type of scaling has an effect that increases with mEPSC amplitude and causes the TTX distribution to diverge from the control distribution, we have named the phenomenon “divergent scaling” to differentiate it from uniform scaling.
IV. SPECIFIC AIM 2

Design and validate a statistical method to compute the generalized mathematical transformation induced by activity blockade of cultured mouse cortical neurons.

Rationale

The rank-order test is currently the most widely-accepted method used to quantify the homeostatic transformation of mEPSC amplitudes but has the disadvantage of being a binary test with only two possible outcomes: either uniform multiplicative scaling is present in the data or it is not. A number of published studies that apply the rank-order method conclude that it does not find uniform scaling in their data; this includes the results of Specific Aim 1 in the current study. Because these conclusions disagree with the expectation that homeostatic plasticity causes uniform multiplicative scaling, it would be useful to know why the method failed to find it in those data and what the scaling transformation actually looks like if it is not uniform. A uniform multiplicative transformation is linear: it has the form $y = mx + b$ with $b = 0$. However, because the additive term $b$ is assumed to be zero, uniform multiplicative scaling is only a subset of all possible linear transformations. A more generalized analysis process, capable of detecting linear transformations in which $b$ can have any value, would be able to detect uniform multiplicative scaling as well as provide diagnostic information in cases where uniform multiplicative scaling is not present.

The precise form of the transformation in mEPSC amplitudes induced by homeostatic plasticity also has implications for the possible mechanisms of homeostatic plasticity, which remain
unknown. Studies attempting to identify the cellular mechanism responsible for homeostatic plasticity have been based on the assumption that homeostatic plasticity causes uniform multiplicative scaling, which implies a cell-wide postsynaptic mechanism, or some other type of mechanism that affects all synapses by the same magnitude. In the face of evidence that homeostatic plasticity may cause non-uniform scaling, there is a need to identify with certainty the actual homeostatic transformation in mEPSC amplitudes, because this informs the type and location of the mechanism responsible.

**Approach**

We will adapt the process of data standardization into a process to compute the mathematical transformation between two distributions. The accuracy of the process will be verified by using a bootstrapping procedure to repeatedly apply the process to two distributions sampled from the control data, which should yield scaling factors that correspond to a transformation of identity. This procedure will also yield a bootstrap distribution of test statistics which we will use to calculate the statistically valid critical value of the Anderson-Darling test on transformed mEPSC amplitude data. As in the rank-order method, we will consider the transformation computed by our process to be accurate and successful if it does not result in a statistically significant difference between the scaled and unscaled distributions. We will also verify that the process computes the expected scaling factors when applied to data in which uniform multiplicative scaling has been simulated, to ensure that uniform multiplicative scaling can be detected if present.

Once our process has been validated, we will apply it to our experimental data and compare the accuracy of the resulting transformation to the transformations computed by the rank-order
and iterative methods. The relative accuracy of the transformations will be judged by eye, and by the p-values of the corresponding Anderson-Darling tests.

**Results**

To devise a generalized method of calculating the transformation between two distributions of mEPSC amplitudes, we began with the concept of data standardization. Data standardization is used to compare two or more samples that differ in scale and are normally distributed: the values in each sample are transformed by subtracting the sample’s mean and dividing by the sample’s standard deviation, so that each sample has a mean of 0 and a standard deviation of 1 (Equation 1). However, we are interested in the mathematical transformation between untreated and treated mEPSC distributions in addition to the similarity of the standardized distributions. Therefore, we used the concept of standardization to derive a method, comparative standardization, for computing a transformation function that, when applied to the control data, produces transformed data with the same μ and σ as the pooled TTX data.

**mEPSC amplitude data can be standardized using a generalized extreme value distribution**

To obtain the μ and σ necessary for standardization, we first attempted to fit the pooled control and TTX data with a normal distribution but found that the fit was very poor (Figure 12A-B, orange lines). However, the pooled data sets are both well fit by a generalized extreme value (GEV) distribution (Figure 12A-B, blue lines). The GEV distribution has parameters μ and σ, which are analogous to the mean and standard deviation of the normal distribution (Figure 12C), and an additional shape parameter ξ, which affects the slope of the distribution tails (Figure 12D). Data that fit a GEV distribution can be standardized using the μ and σ of the distribution, but GEV distributions with equal μ and σ are only equivalent if the ξ parameters are also equal.
Two standardized GEV distributions with the same shape parameter are thus equivalent.

**The comparative standardization process**

Comparative standardization is a method of computing the linear mathematical transformation between two sample distributions. The requirements of comparative standardization are that the samples must be fit by the same parent distribution (e.g., a generalized extreme value distribution), and that they must be able to be standardized. Given two samples GEV₁ and GEV₂ that are both fit by a generalized extreme value distribution, standardizing both samples gives them the same location and scale parameters (0 and 1, respectively; Equation 3a-b). This allowed us to set them equal and solve algebraically for the second sample GEV₂ as shown in Equation 3c, which yields an equation that linearly transforms the first sample GEV₁ to fit the second sample GEV₂ by applying a multiplicative factor \( f_{\text{mult}} \) and additive factor \( f_{\text{add}} \) (Equation 3d). A stepwise illustration of this process shows that the multiplicative factor transforms the scale, or width, of the sample (Figure 13B) while the additive factor centers it at the correct position (Figure 13C). The transformed GEV₁ can then be statistically compared to GEV₂; if the transformation function accurately recapitulates the mathematical relationship between the two samples, the transformed GEV₁ will be statistically indistinguishable from GEV₂ and the method is considered successful. The transformation calculated by comparative standardization is linear, as the transformation function has the form \( y = mx + b \), and uniform in that the same factors are applied to every value in the transformed sample. However, the transformation is not necessarily uniformly multiplicative in the sense that every sample value is modified by the percentage: a non-zero additive factor will cause the apparent magnitude of the transformation to vary with the magnitude of the sample values. This concept is illustrated in the next section.
Different linear transformations are detectable using comparative standardization

Because of the inclusion of an additive factor, comparative standardization has several distinct possible outcomes when used to compute the transformation between an untreated distribution of mEPSC amplitudes and mEPSC amplitudes recorded in cells after inducing homeostatic plasticity. The homeostatic amplitude increase will result in a multiplicative factor greater than one, leaving three possibilities based on the value of the additive factor, which can be less than zero, equal to zero, or greater than zero. These three possible outcomes are illustrated in Figure 14 by taking a data sample GEV1 and applying three scaling transformations, each with the same multiplicative factor of 1.25 combined with a negative, zero, or positive additive factor. An additive factor less than zero produces a scaled amplitude CDF that overlaps with the unscaled data in the smallest amplitudes and diverges at larger amplitudes (Figure 14A), which is similar to our experimental data (Figure 4C). A plot showing the ratio of scaled to unscaled amplitudes as a function of the unscaled amplitudes confirms this similarity: the ratio increases with increasing amplitude (Figure 14B), similar to the ratio of TTX-treated to control amplitudes that was observed in our data (Figure 11D). An additive factor of zero results in uniform multiplicative scaling with an evenly right-shifted scaled CDF and a constant ratio of scaled to unscaled amplitudes (Figure 14C-D), while an additive factor greater than zero results in “convergent” scaling characterized by a large amount of separation in the smallest amplitudes of the scaled and unscaled amplitude CDFs and a ratio that decreases with increasing amplitude (Figure 14E-F).

Application of comparative standardization to homeostatic plasticity data

To apply comparative standardization to homeostatic plasticity data, the requirement that the control and TTX-treated distributions of mEPSC amplitudes have equal ξ must first be met, to ensure that the distributions are equivalent when standardized. To accomplish this, the control
and TTX distributions are combined and a GEV distribution is fit to the combined data to obtain a value $\xi_{\text{shared}}$. To fit the separate control and TTX GEV distributions, the $\xi$ parameter is constrained to $\xi_{\text{shared}}$. The $\mu$ and $\sigma$ from the fitted distributions can then be substituted into Equation 3d as shown in Equation 4, yielding a multiplicative and an additive factor that transform the control data to fit the GEV distribution of the TTX data as in Figure 13. The transformed control data is then statistically compared to the TTX data with a test for the equivalence of distributions, as in the rank-order and iterative methods. If the transformation function accurately recapitulates the mathematical relationship between mEPSC amplitudes recorded in control neurons and amplitudes recorded in TTX-treated neurons, the transformed control data will be visually similar and statistically indistinguishable from the TTX data, and this will be considered a successful result.

In addition to the issues previously discussed in Aim 1, the Kolmogorov-Smirnov test has the drawback of being most sensitive to deviations in the center of the distributions. The Anderson-Darling test is another test for the equivalence of distributions that has the same requirements as the K-S test but is more sensitive to deviations in the tails of the distributions (Scholz and Stephens 1987). We believe that this attribute makes the A-D test better suited for use on homeostatic plasticity data and use A-D for the remainder of this study, when not comparing our results to previous studies.

**Comparative standardization computes the expected scaling factors in unscaled data**

We performed a bootstrapping procedure for validation, both to ensure that comparative standardization accurately computes the mathematical transformation between two distributions of mEPSC amplitudes and to calculate the test statistic distribution necessary to determine the critical value of the Anderson-Darling test statistic in our data. Two distributions
of events were repeatedly resampled (10,000 iterations) from our untreated control cells, comparative standardization was used to calculate the mathematical transformation between the first distribution and the second, then the first distribution was transformed and compared to the second with an A-D test. The resulting bootstrap distributions of multiplicative and additive factors had means of 0 and 1, respectively, as expected (Figure 15A-B), and the width of both distributions was narrower than the bootstrap distributions of slope and intercept coefficients from the rank-order method (Figure 10A-B), indicating that comparative standardization is not only accurate, but more precise than the rank-order method. The bootstrap distribution of the A-D test statistic was used to obtain a critical value by computing the 95th percentile (for a significance level of 0.05) of the distribution, resulting in a critical value of 0.009 (Figure 15C).

**Comparative standardization computes the expected scaling factors on uniformly scaled data**

We repeated the bootstrapping procedure described above with the additional step of multiplying the second distribution in each iteration by a factor of 1.25, thus creating a simulated uniform-multiplicative homeostatic plasticity effect in a manner similar to the empirical simulations described in Aim 1. This comparison was expected to yield an average multiplicative factor of 1.25 and an average additive factor of 0. The bootstrap distributions showed that, on average, comparative standardization calculated the expected value of both factors (Figure 16), confirming that the process is capable of detecting uniform multiplicative scaling and that data that exhibit uniform multiplicative scaling will result in an additive factor of 0.
**Comparative standardization finds divergent scaling in experimental data**

Finally, comparative standardization was applied to our experimental data: to obtain the value of a shared $\xi$ parameter, the control and TTX mEPSC amplitude distributions were combined and the combined data were fit with a generalized extreme value distribution (Figure 17A). The control and TTX data were then fit with separate GEV distributions with the $\xi$ parameter constrained to $\xi_{\text{shared}}$ (Figure 17B-C), which resulted in no change from the $\mu$ and $\sigma$ parameters of the GEV distributions that were fit with unconstrained $\xi$ (Figure 12A-B). Using the $\mu$ and $\sigma$ parameters of the control and TTX GEV distributions, the multiplicative and additive scaling factors were calculated as described above. The control data were transformed with these factors ($f_{\text{mult}} = 1.39$, $f_{\text{add}} = -1.80$; Equation 4) and the transformed data were compared to the TTX data using an Anderson-Darling test, which yielded a standardized test statistic of -0.163 (Figure 17). Because the observed test statistic does not exceed the critical value of 0.009 (Figure 15), the null hypothesis that the two distributions are equivalent is not rejected, leading to the conclusion that comparative standardization successfully computed the transformation between control and TTX data with a multiplicative factor of 1.39 and an additive factor of -1.80.

A negative additive component is indicative of divergent scaling (Figure 14A-B), which supports our earlier observation that our data are divergently scaled. Because this runs counter to the current understanding of homeostatic plasticity as a uniform multiplicative transformation, we used another bootstrapping procedure to evaluate the likelihood of this result. For each iteration of this procedure, one set of mEPSC amplitudes was sampled from untreated control neurons and another set was sampled from TTX-treated neurons; comparative standardization was used to transform the control sample to fit the TTX sample. The procedure was repeated for 10,000 iterations and produced bootstrap distributions of multiplicative and additive factors (Figure 18). The distribution of additive factors showed that an additive factor of 0, and thus
uniform multiplicative scaling, is extremely unlikely, as an additive factor close to 0 occurred in less than 1% of the bootstrap iterations.

**Comparative standardization results in a closer match than the rank-order and iterative methods**

To directly compare results from all three methods – rank-order, iterative, and comparative standardization – we replotted the results of the first two methods by using their scaling factors to scale the control data up to match the TTX data (Figure 19), rather than downscaling the TTX data as was shown previously in Figure 7 and Figure 9. This was done to match the direction of the scaling in comparative standardization. For the iterative method, the control data were upscaled, the smallest upscaled control amplitude was taken as the “threshold,” and all mEPSC amplitudes smaller than this value were discarded from the TTX data. Although the p-values computed directly by the Anderson-Darling test cannot be used to judge statistical significance, they are still an accurate measure of relative goodness of fit across instances of the test with similar sample sizes. Here we use the A-D p-values (not the values calculated using the bootstrapped test statistic distribution) as a quantitative measure to compare the goodness of fit of the transformations computed by the different methods on the same data. Note that these are not the same p-values previously reported in Figure 7 and Figure 9 because those values were obtained with the Kolmogorov-Smirnov test, not Anderson-Darling. Of the three methods, comparative standardization computes the transformation that fits the control data most closely to the TTX data, judging both by the A-D p-values and by eye: the transformation of the rank-order method has $p = 3.75 \times 10^{-13}$ and produces a good fit of the scaled control data to TTX in the middle but poor alignment at either tail. The iterative method aligns the scaled data slightly better than the rank-order method in the smaller quantiles but produces greater deviation in the upper quantiles, with $p = 6.18 \times 10^{-6}$. Comparative standardization computed a
transformation that produced a close alignment of the scaled control data to TTX through the entirety of the distributions with $p = 0.429$, which was the best match overall.

**Discussion**

Using the concept of data standardization, we have developed a new method to quantify the mathematical transformation between two distributions of mEPSC amplitudes. The new method, comparative standardization, was validated using a bootstrapping procedure to demonstrate that it computed the expected transformation on both unscaled data and data that had been scaled by a uniform multiplicative factor. The bootstrapping procedure was also used to estimate the distribution of the Anderson-Darling test statistic on the transformed data, allowing us to compare the transformed distributions with statistical validity. When applied to our experimental data, comparative standardization computed a divergent scaling transformation, with a negative additive factor, that resulted in no significant difference between the scaled distribution and the TTX distribution. We compared the transformation computed by comparative standardization to the transformations computed by the rank-order and iterative methods and found that the divergent transformation computed by comparative standardization resulted in the closest match between scaled and TTX. The goodness of fit between the scaled control mEPSC amplitudes and the TTX amplitudes is a measure of how well the transformation recapitulated the effects of homeostatic plasticity and reproduced them mathematically in the untreated control data; the fact that the divergent scaling transformation computed by comparative standardization produces a closer fit than the uniform multiplicative scaling transformations computed by the other methods is strong evidence in support of our conclusion that our data exhibit divergent scaling, not uniform multiplicative scaling.
Unlike the rank-order and iterative methods, comparative standardization can detect three different types of linear scaling transformation: uniformly multiplicative, divergent, and convergent. The type of the transformation depends on whether the additive factor is zero, negative, or positive, respectively. Although all three transformations are mathematically uniform in that the same scaling factors are always applied to every mEPSC amplitude, only a transformation with an additive factor of zero, which corresponds to what is known as uniform multiplicative scaling, has a uniform effect on the data. The divergent transformation will have a proportionately smaller effect on the smallest amplitudes, because the additive factor represents a larger proportion of their magnitude. That is, an additive factor of -3 will decrease a mEPSC with an amplitude of 6 pA by 50%, resulting in a proportionately larger effect than on a mEPSC with a 12 pA amplitude, which is only decreased by 25%. For clarity, and to maintain continuity with previous work, we will continue to refer to a scaling transformation with an additive factor of zero as “uniform multiplicative scaling,” with the understanding that “uniform” applies to the “multiplicative” aspect, as all three types of transformation are mathematically uniform.

Comparative standardization has several advantages over the rank-order and iterative methods. The first and most important benefit is that it is not constrained to detecting only uniform multiplicative scaling but can detect and identify divergent and convergent scaling as well. This aspect of comparative standardization makes it a useful tool for assessing the scaling transformation in data sets that do not exhibit uniform multiplicative scaling per the rank-order and iterative methods. Additionally, the linear regression fit used by the rank-order method requires that the treated and untreated data have the same sample size, requiring an additional layer of processing on the data that has the potential to result in sampling error if events are randomly discarded from the larger sample, or overfitting if quantile sampling is used (to obtain
equal-sized samples for the rank-order method, quantile sampling computes as many quantiles per cell as there are cells in the other experimental group, potentially resulting in a very large sample size if the number of cells per group is large; see Methods for details). Because comparative standardization computes its transformation from the parameters of a distribution fit to the data, there is no constraint on the relative size of the samples and these potential issues are avoided. Lastly, we believe that the transformation computed by comparative standardization will produce a closer match between the scaled and treated data than the rank-order or iterative methods because it includes an additional parameter. We have shown that this is the case in our data; we will examine whether a closer match is also found in other data sets in Specific Aim 3. Comparative standardization does have the drawback that the transformation it computes is somewhat dependent on how well the data are fit by the chosen distribution. If one or both of the data samples are not well fit, it is likely that the transformation will be less accurate and result in a poorer match than for data that are well fit. Interpretation of comparative standardization’s results on data that are poorly fit should be adjusted accordingly.

In addition to its applicability to homeostatic plasticity data, comparative standardization can be applied to any pair of data samples provided that they meet the requirements of the process: the samples must fit the same parent distribution, the parent distribution must have location and shape parameters, and, in the case of distributions with additional parameters, those parameters must be handled such that data fit by that parent distribution are equivalent when standardized.

An additional point of interest is raised by the fact that mEPSC amplitude distributions are well fit by a generalized extreme value distribution. This type of distribution is typically used to describe the distribution of the maximum values in a population of normal distributions. If this
aspect of the GEV distribution also applies to mEPSC amplitude, it raises two questions about synaptic physiology: Are the mEPSCs that are detectable at the soma the maxima of multiple larger distributions of synaptic events? If so, then what do those larger, underlying distributions represent? One potential way to address these questions is by using immunohistochemistry and fluorescence imaging to visualize synaptic sites on the dendritic arbor of neurons. The size of many synapses could be collected and analyzed with a gaussian mixture model, which attempts to fit data with multiple normal distributions, or another clustering method suitable for testing data for underlying distributions.
V. SPECIFIC AIM 3

Demonstrate that comparative standardization computes a divergent transformation on additional data sets, and that this transformation results in a better match between treated and untreated mEPSC amplitudes than the uniform multiplicative transformation computed by the rank-order and iterative methods.

Rationale

In Specific Aim 1, the results of the rank-order and iterative methods on data from a simulation of uniform multiplicative scaling demonstrated that these methods were capable of finding a very close match in data that exhibit uniform scaling. However, published studies that have applied these methods to homeostatic plasticity data often do not obtain as close a match as was seen in the simulation data, suggesting that the data may actually be non-uniform. Additionally, several homeostatic plasticity studies have concluded that their data are not uniformly scaled, and a non-canonical preparation (i.e. one that does not use neonatal rat cortical neurons as was done in (Turrigiano et al 1998)) is often cited as the reason. We wish to investigate whether uniform multiplicative scaling is as ubiquitous as previously believed, and whether experimental preparation has any bearing on whether homeostatic plasticity induces a uniformly multiplicative or non-uniform increase in mEPSC amplitudes.

We have developed a novel method for computing the homeostatic transformation between treated and untreated distributions of mEPSC amplitudes and used it to show that our data, recorded in mouse cortical neurons treated with TTX, exhibit divergent scaling, not uniform
multiplicative scaling. However, because the method was developed and validated using only a single data set, it is possible that this finding is a result of overfitting, or that it is otherwise unique to our data. For these reasons, comparative standardization should be tested on other data sets recorded under different experimental conditions to ensure that it can compute an accurate transformation, and to determine whether data other than our own exhibit divergent scaling. Applying comparative standardization to additional data sets will also allow us to better assess whether this method consistently finds a more accurate transformation than the rank-order and iterative methods.

**Approach**

We will apply the rank-order and iterative methods to three previously published data sets recorded in a variety of species and brain regions, and compare the results to data from our empirical simulations of uniform multiplicative scaling to determine whether the methods find as close a match in the experimental data as in the simulation data. If the transformation computed by either method is not as accurate as the transformations computed on the simulation data, this will be taken as evidence that the experimental data do not exhibit uniform scaling. Ratio plots will be generated for the three new data sets, and the ratio plots will be compared across all four sets of experimental data in an attempt to determine whether experimental conditions such as species, brain region, or drug treatment have an identifiable effect on the ratio of treated to control mEPSC amplitudes.

Next, we will apply comparative standardization to the three additional data sets. The transformation computed by comparative standardization will be compared to the transformations computed by the rank-order and iterative methods to determine which method results in the most accurate transformation, as measured by Anderson-Darling p-value and
visual examination. If comparative standardization results in the largest p-value and closest visual match in all three data sets, our hypothesis that comparative standardization finds the most accurate transformation is strengthened. The additive factor computed from each data set will be used to determine whether the data exhibit divergent scaling or uniform multiplicative scaling. A negative additive factor, indicative of divergent scaling, would support our hypothesis that divergent scaling is not unique to mouse cortical neurons.

**Results**

To determine whether the presence of divergent scaling is unique to the current experimental conditions—relatively mature (13-14 DIV) dissociated mouse cortical cultures—we examined previously published data from other laboratories: 12-15 DIV dissociated mouse hippocampal cultures treated with TTX for 48 hours (Altimimi and Stellwagen 2013), 10-12 DIV dissociated rat cortical cultures treated with TTX for 24 hours (Fong *et al* 2015), and 10-12 DIV dissociated rat cortical cultures treated with CNQX for 24 hours (Fong *et al* 2015). This set of experiments provides examples of homeostatic plasticity across multiple species (rat and mouse), brain regions (cortical and hippocampal), and drug treatments for inducing homeostatic plasticity (action potential blockade via TTX and receptor inhibition via CNQX).

**The rank-order method finds uniform multiplicative scaling in one of three additional data sets**

We created quantile-sampled CDFs of control and treated data for all three data sets and applied the rank-order method to scale the treated data to control (Figure 20; dashed vermillion line in Figure 20A, C, E). The CDFs and rank-ordered plots differed from the previously published plots only in their variability (for comparison see Figures 3A, B in (Altimimi and Stellwagen 2013); Figures 3D, E; Supplementary Figure 3A in (Fong *et al* 2015)). As previously reported,
scaling of the treated data by the coefficients of the linear regression fits to the ranked data produced a scaled CDF roughly similar to the CON CDF in all data sets (Figure 20A, C, E; dashed vermilion and solid black lines). However, it is notable that, while the rat cortical CNQX data ($N_{con} = 2670, N_{cnqx} = 2820; p = 0.13$, K-S test) show a very good fit, both the rat cortical TTX data ($N_{con} = 1410, N_{ttx} = 1740; p = 2.9 \times 10^{-7}$, K-S test) and the mouse hippocampal TTX data ($N_{con} = 540, N_{ttx} = 600; p = 7.7 \times 10^{-3}$, K-S test) sets exhibit some deviation, particularly visible in the lower quantiles of the data (insets, Figure 20A, C, E). Based on the results of the rank-order method on our simulation data (Figure 6), we expect the process to produce a nearly perfect fit if the scaling relationship is truly uniformly multiplicative and the detection threshold is small (~5 pA), and a very good fit even if the detection threshold is high (~7 pA). The failure of the rank-order process to produce as good a fit as on the simulation data calls into question whether the rat cortical TTX or the mouse hippocampal TTX data exhibit uniform multiplicative scaling.

The iterative method finds uniform multiplicative scaling in none of the additional data sets

To further examine whether the previously published data show uniform multiplicative scaling, we next applied the iterative method (Kim et al 2012) as shown in Figure 8 and Figure 9. The transformation computed by this process failed to produce a good fit in any of the three data sets: both the rat cortical TTX data ($N_{con} = 1410, N_{ttx} = 1715; p = 2.4 \times 10^{-4}$, K-S test) and the rat cortical CNQX data ($N_{con} = 2670, N_{cnqx} = 2750; p = 6.2 \times 10^{-5}$, K-S test) yielded transformations that produced poor matches (Figure 21A-D) compared to the expected results on uniformly scaled data (Figure 8). While the transformation in the mouse hippocampal data ($N_{con} = 540, N_{ttx} = 318; p = 0.76$, K-S test) appeared to produce a close match, closer examination reveals that this was achieved through the use of a large scaling factor which caused the removal of roughly half of the TTX distribution due to being under the detection threshold set by the smallest control mEPSC amplitude (Figure 21E-F). These results suggest that non-uniform scaling may be
a common outcome of homeostatic plasticity and not unique to our data or experimental conditions.

**Ratio plots show divergent scaling in all additional data sets**

We generated ratio plots of each of the previously published data sets in order to examine their scaling relationships as a function of mEPSC amplitude and verify that all three exhibit non-uniform scaling. Despite being most closely related to the canonical preparation (Turrigiano *et al* 1998), the data recorded in rat cortical cultures treated with TTX show divergent scaling, with the ratio of TTX to control amplitudes continuing to increase over nearly the entire data range (Figure 22A). The data from rat cortical cultures treated with CNQX and from mouse hippocampal cultures treated with TTX are also divergent, with their ratio increasing over at least the first three quartiles of the data range (Figure 22B-C). In order to compare and contrast the ratio plots from all four sets of experimental data, we replotted them, this time using the same y-axis range on all four for consistency (Figure 23). When the data are viewed in this way, the wide variation among the four data sets is clearly apparent: the slope of the increasing ratio, as well as the location and presence of a plateau, vary across all four. The only similarity, aside from the fact that all four data sets exhibit divergent scaling, is that the data sets whose ratios reach a plateau (rat cortical CNQX, mouse cortical, and mouse hippocampal) all do so around roughly the third quartile of the data. From these findings, we conclude that divergent scaling is not unique to our data set but may in fact be a more common outcome of homeostatic plasticity than uniform multiplicative scaling.

**Comparative standardization finds divergent scaling in all additional data sets**

To confirm and quantify the presence of divergent scaling in the three additional data sets, we applied comparative standardization to each of them. In all three data sets, the data were well-
fit by generalized extreme value distributions with a shared shape parameter (Figure 24A-B, Figure 25A-B, Figure 26A-B) and thus met the requirements for the comparative standardization process to be valid. The data from rat cortical neurons treated with TTX showed deviation between the TTX and scaled control distributions from all three methods but comparative standardization produced the closest match, with good alignment up to approximately the 70\textsuperscript{th} percentile, after which the deviation becomes visually apparent (rank-order method, \( p = 7.13 \times 10^{-10} \), iterative method, \( p = 5.24 \times 10^{-10} \), comparative standardization, \( p = 0.0223 \); Figure 24C-E).

In the rat cortical CNQX data, the iterative method showed marked deviations (\( p = 9.59 \times 10^{-9} \); Figure 25D) and the rank order method produced a good transformation that resulted in \( p = 0.0884 \) and very little visible deviation (Figure 25C), but comparative standardization yielded a transformation with \( p = 0.68 \), which was visually a near-perfect fit between the scaled control and CNQX data (Figure 25E). Neither the rank order (\( p = 0.0004 \)) nor the iterative method produced a good transformation in the mouse hippocampal neurons; although the transformation from the iterative method yielded a high \( p \)-value (\( p = 0.783 \); Figure 26D), the corresponding scaling factor was so high that roughly half the TTX data were discarded as subthreshold. Comparative standardization, by contrast, produced a transformation with \( p = 0.99 \) and a good fit by eye (Figure 26E). Based on these findings, our hypothesis – that comparative standardization computes a divergent transformation on the additional data sets, and that this transformation results in a better match between treated and untreated mEPSC amplitudes than the uniform multiplicative transformation computed by the rank-order and iterative methods – is supported.
The multiplicative factor from comparative standardization is strongly correlated with the additive factor.

The fact that comparative standardization uses two mathematical factors to compute the transformation in mEPSC amplitudes induced by homeostatic plasticity raises the question of whether the additional factor represents a separate and additional biological effect. A scatter plot of both factors from all four data sets (Figure 27) shows a strong negative correlation between the multiplicative and additive factors: as the multiplicative factor increases, the additive factor becomes more negative. While this correlation does not completely rule out the existence of multiple homeostatic plasticity effects, the strength of the correlation makes it unlikely.

Comparison of the scaling factors from the rank-order method and comparative standardization

We wanted to determine whether comparative standardization was simply calculating a more accurate version of the transformation detected by the rank order method. To compare the two methods, we plotted the slope coefficient from the rank order method against the multiplicative factor from comparative standardization (Figure 28A) and the intercept coefficient against the additive factor (Figure 28B) for all four data sets. At first glance, neither set of factors appears to be correlated, but closer examination reveals that a strong correlation between the factors from the rank order method and those from comparative standardization is disrupted only by the values of the transformation in the rat cortical TTX data; the remaining data sets show correlation.
The effects of truncation on the results of the rank-order method and comparative standardization

To test the claim made in other publications that the intercept coefficient in the rank order method corrects for an experimental detection threshold, we plotted both the slope and the intercept coefficients as a function of threshold for the four data sets (Figure 29A-B). Surprisingly, both coefficients show a correlation with threshold in all data sets except the rat cortical neurons treated with TTX, indicating that the intercept is unlikely to be an effective correction for the effects of a detection threshold because the threshold also affects the slope coefficient. This result suggests that a detection threshold may cause the magnitude of the homeostatic plasticity effect, as measured by the rank order method, to be artificially inflated; to determine whether comparative standardization was also subject to artificial inflation, we next plotted the multiplicative factors and additive factors as a function of detection threshold for all four data sets. The three data sets from the empirical simulations were also included in these plots to serve as an example of how a detection threshold affects the comparative standardization factors when the transformation is known (Figure 30A-B). The multiplicative factors from the simulation data increase slightly with the simulated detection threshold, from about 1.3 in the untruncated data to 1.4 in the data with a threshold of 7 pA, indicating that a 7 pA threshold can cause inflation of the multiplicative factor by roughly 0.1. In contrast, the multiplicative factors in the experimental data reach values greater than 2; it is unlikely that the artificial inflation due to detection threshold is responsible for the full magnitude of the multiplicative factors. A similar trend was observed in the additive factors: a detection threshold of 7 pA cause the additive factor in the simulation data to increase in magnitude by about 2, but the additive factors in the experimental data are much larger. We conclude that, although the homeostatic transformation in mEPSC amplitude can be slightly overestimated by comparative
standardization in data with a high detection threshold, the magnitude of the overestimation is small relative to the magnitude of the transformation.

Discussion

We acquired three additional experimental data sets from our collaborators. All additional data sets were performed using dissociated neuronal cultures; the first data set was recorded in rat cortical neurons treated with TTX, the second was recorded in rat cortical neurons treated with CNQX, and the third was recorded in mouse hippocampal neurons treated with TTX. We applied the rank-order test to the additional data sets and found that, based on the results of the rank-order test on our simulation of uniform multiplicative scaling, only the transformation computed on the rat cortical CNQX data resulted in a match close enough to conclude that the transformation was accurate. The transformations computed on the other two data sets were reasonably close by eye, but did not produce the nearly perfect match found in the simulation data, a result that does not support uniform multiplicative scaling in those data. The iterative method failed to detect uniform scaling in any of the three data sets; all three transformations resulted in greater deviations between the scaled and control distributions than were expected based on the results of the iterative method on the simulation data. These findings are evidence that all three of these data sets may exhibit non-uniform scaling, although the success of the rank-order method on the rat cortical CNQX data makes the evidence weaker for non-uniform scaling in those data than in the other data sets.

Ratio plots of the three additional data sets show an increasing ratio of treated to control mEPSC amplitudes, which is indicative of divergent scaling. The presence of a non-constant ratio in the rat cortical CNQX data was a surprising result, given that the outcome of the rank-order method on those data was consistent with uniform multiplicative scaling, and suggests that the
rank-order method is prone to error in the form of false positives. When the ratio plots of all four experimental data sets (our mouse cortical TTX data plus the three additional data sets) are viewed together, it is clear that, although all four ratios increase with control mEPSC amplitude, the trends in the ratios are very different across the four data sets. This provides no conclusive evidence for or against the idea that different experimental conditions might produce different homeostatic scaling transformations: while there is no clear commonality among the ratio plots of data from the same species or brain region, the possibility remains that each unique combination of species, brain region, treatment, etc. may have a characteristic transformation. The analysis of many more data sets, with duplicates of the same or very similar experimental conditions, is required to either prove or disprove this possibility.

The control and treated mEPSC amplitudes from all three of the additional experimental data sets were well fit by a generalized extreme value distribution and thus met the requirements for comparative standardization to be valid. We applied comparative standardization to the three data sets and found that a negative additive factor was computed for all three, which is indicative of divergent scaling and supports our hypothesis that the three additional data sets exhibit a divergent scaling transformation. Comparative standardization also found the closest match between the control and treatment distributions across all data sets, even in the rat cortical CNQX data, in which the rank-order method appeared to find uniform multiplicative scaling. These findings support our hypothesis that comparative standardization computes a transformation that more closely recapitulates the effect of homeostatic plasticity on mEPSC amplitudes than other methods, and that the transformation is divergent.

The results of applying the rank-order method to data from empirical simulations of uniform multiplicative scaling in Specific Aim 1 suggested that the goodness of fit required to conclude
that data exhibit uniform multiplicative scaling is more stringent than previously believed, which could lead researchers to conclude that their data exhibit uniform scaling when the data are actually non-uniform. Our findings in the current aim confirm this: the three additional data sets analyzed in Specific Aim 3 were all identified exhibiting uniform multiplicative scaling in their original publications, but our ratio plots and comparative standardization results show that all three exhibit divergent scaling. If these three studies are taken as a representative example, it is possible that other published data sets were also inappropriately found to be uniformly multiplicative. Interestingly, the data set recorded in rat cortical neurons with CNQX treatment did meet the stringent goodness-of-fit requirements set by the simulations, but were still shown to be divergently scaled by both an increasing ratio of treated to untreated mEPSC amplitudes and a negative additive factor from comparative standardization. This finding – that the rank-order method can generate a false positive by computing an accurate transformation in divergently-scaled data – suggests that Kim et al were correct when they pointed out that the rank-order method does not compute a purely multiplicative transformation. The finding also clearly demonstrates an advantage of comparative standardization over the rank-order method: where the rank-order method is based on the assumption that any accurate transformation is uniformly multiplicative, comparative standardization unambiguously identifies what type of scaling transformation has been computed, making misclassification impossible if an accurate transformation is found.

The comparison among the different methods for computing a homeostatic transformation also uncovered a potential drawback of the iterative method: in the mouse hippocampal TTX data, the scaling factor that produced the best fit was large enough that nearly half of the TTX-treated data were discarded because, when downscaled by that factor, they fell below the smallest control amplitude. Additionally, the range of scaling factors that formed the “peak” of p-values
was wider than in the other data sets by roughly a factor of 10 (compare Figure 21E to Figure 21A, Figure 21B, and Figure 9A; note x-axis range), indicating that the iterative method was far less precise on the mouse hippocampal TTX data than on the other data sets. We believe that both of these occurrences were caused by the fact that the Kolmogorov-Smirnov test loses sensitivity drastically with decreasing sample size. When applying the iterative method, larger scaling factors will always result in a smaller sample size, as more events are discarded from the TTX distribution. In the mouse hippocampal data specifically, this resulted in a wide range of large scaling factors that produced relatively good p-values by discarding an improbably large proportion of the TTX data, thus decreasing the sample size and the test’s sensitivity. We suspect that this may be more likely to occur on data where the scaling transformation has a large additive factor: as the magnitude of the additive factor increases, so does the deviation of the transformation from uniform multiplicative scaling, making it increasingly difficult to find a single multiplicative factor that produces a good fit. The mouse hippocampal data had the largest additive factor of all four experimental data sets by far, which supports this theory.

Performing comparative standardization across multiple experimental data sets also allowed us to do some limited investigation into what the method could tell us about the homeostatic plasticity effect on mEPSC amplitudes. The presence of a non-zero additive factor in a comparative standardization transformation represents the degree to which the data converge or diverge with increasing amplitude, but it is unclear whether the divergence represented by the negative additive factor in homeostatic transformations is related to the amplitude increase, or whether it is a separate effect. To answer this question, we looked at the multiplicative and additive factors computed by comparative standardization across all four data sets. Figure 27 shows that the additive and multiplicative factors are strongly negatively correlated. The strength of this correlation makes it unlikely that the divergence represented by the additive
factor is a separate effect from the homeostatic amplitude increase, but instead suggests that both factors increase in magnitude with the strength of the homeostatic effect.
VI. DISsertation Summary

Conclusions by specific aim

Specific Aim 1

• A homeostatic plasticity data set recorded in dissociated mouse cortical cultures and treated with TTX does not exhibit uniform multiplicative scaling.

Specific Aim 2

• A novel method, comparative standardization, accurately computes the mathematical transformation between two sets of mEPSC amplitudes and can distinguish between uniform-multiplicative, divergent, and convergent scaling.

• Comparative standardization finds divergent scaling in the mouse cortical TTX data.

• Comparative standardization computes a more accurate transformation than the rank-order or iterative method on the mouse cortical TTX data.

Specific Aim 3

• Homeostatic plasticity data sets recorded in rat cortical neurons treated with TTX, rat cortical neurons treated with CNQX, and mouse hippocampal neurons treated with TTX exhibit divergent scaling, not uniform multiplicative scaling.

• Comparative standardization computes a more accurate transformation than the rank-order or iterative methods on all four experimental data sets.
Discussion

This study has demonstrated, based on multiple lines of evidence and rigorous validation, that the uniform multiplicative scaling hypothesis is an insufficient model of homeostatic synaptic plasticity across multiple experimental data sets and experimental conditions. Instead, we have proposed the alternative hypothesis that homeostatic plasticity causes divergent scaling, a linear transformation characterized by a multiplicative factor greater than one and an additive factor less than zero. This hypothesis is supported by the results of a new tool, comparative standardization, that computes the linear transformation between two sample distributions and classifies the transformation based on the sign of the additive factor. The transformation computed by comparative standardization more closely recapitulated the homeostatic effect on mEPSC amplitudes than the transformation computed by the rank-order or iterative methods on all data sets that were evaluated, leading us to conclude that it is an overall improvement over these previous methods.

The uniform multiplicative scaling hypothesis of homeostatic plasticity was proposed as an explanation for how networks of neurons in the CNS adapt to continuously-varying levels of input without losing the information stored as variations in relative synaptic strength, and without succumbing to runaway excitation or inhibition. We believe that the divergent scaling accomplishes the same goals as well as, and in some cases better than, uniform multiplicative scaling. The basic premise of homeostatic plasticity, illustrated in Figure 1, is that as network firing activity goes down, individual synaptic strengths go up, and vice versa. Uniform multiplicative scaling would accomplish this compensatory increase in synaptic strengths by increasing the amplitude of each mEPSC by the same percentage of its original amplitude; the relative synaptic weights across the network are perfectly preserved because all synapses are
affected equally. However, all synapses are not equal; some have undergone more potentiation than others and are thus responsible for a greater portion of the information stored in the network, while others may have undergone no potentiation at all. An equal, global increase in the strengths of all synapses strengthens those unpotentiated, naïve synapses along with the stronger potentiated synapses. Divergent scaling, on the other hand, has little effect on the very smallest synapses because its effect is an increasing function of synaptic strength: larger synapses, which are more likely to be important to the function of the network, see a greater homeostatic increase than smaller synapses, such that the relative synaptic weights in the network shift slightly, but their ranking is preserved instead. Because strong synapses are strengthened while the weakest synapses change very little, divergent scaling expands the range of the synaptic weights in the network, thereby improving the signal to noise ratio by increasing the separation between the largest synapses and the smallest. Note that this does not mean that divergent scaling only affects the largest synapses. The amplitude increase is graded across the entire range of mEPSC amplitudes such that all events see at least a slight increase (see ratio plots in Figure 23 – even the smallest control amplitudes correspond to a ratio greater than one). Divergent scaling is thus capable of preventing runaway excitation and inhibition in the same way as uniform multiplicative scaling, but may also serve to reinforce the differential synaptic weights upon which a neuronal network’s function is based.

The divergent scaling model of homeostatic plasticity has a different set of implications for the possible mechanism of homeostatic plasticity than the uniform scaling hypothesis. Uniform scaling requires a mechanism that affects all synapses cell-wide in the same proportion to their original strength. Divergent scaling may also be mediated by these types of mechanisms, but only in conjunction with an additional mechanism whose effect increases with the initial strength of the synapse. The set of possible molecules is thus expanded to include those present
in proportionally greater amounts in larger synapses, or those that require transport to the synaptic sites, such as proteins that are synthesized at the soma rather than locally in the dendrite.

Although homeostatic plasticity refers to changes in synaptic strength in response to both decreased and increased network activity, this study was entirely on the homeostatic increase in mEPSC amplitudes in response to network silencing. The uniform multiplicative scaling hypothesis applies to scaling in both directions; based on our findings here, we predict that divergent scaling also occurs when synapses are scaled down in response to increased network activity. Specifically, we predict that increased network activity will result in a divergent downscaling transformation consisting of a multiplicative factor less than one and a positive additive factor, the reverse of the multiplicative factor greater than one and negative additive factor that are seen in divergent upscaling. An example of this proposed transformation is shown in Figure 31 along with an example of divergent scaling-up. The CDFs of the downscaled data (Figure 31C) demonstrate that, as in upward divergent scaling, downward divergent scaling produces an effect that increases with mEPSC amplitude, causing the scaled CDF to overlap with the unscaled data in the smallest amplitudes and diverge, this time in the downward direction, as amplitude increases.

The improved model of homeostatic plasticity described in this study suggests a process by which homeostatic plasticity enhances the capacity of a neuronal network for information processing in addition to its function of stabilizing network activity, and improves our understanding of how different forms of synaptic plasticity interact to produce a functional nervous system.
VII. FIGURES

Figure 1. Diagram of the homeostatic plasticity effect in terms of network activity and mEPSC amplitude.
Figure 2. Flow diagram of a bootstrapping procedure for validating the rank-order method.
Figure 3. The amplitude of miniature excitatory post synaptic currents (mEPSCs) is increased in data previously recorded in dissociated cultures of mouse cortical neurons treated with 500 nM tetrodotoxin (TTX) for 48 hours. (A) Image of typical pyramidal cell. (B) Representative voltage clamp recordings from a TTX-treated neuron and an untreated control neuron. (C) Miniature excitatory post-synaptic currents averaged from the recordings in (B). (D) Grand mean of mEPSC amplitudes recorded in control and TTX-treated neurons (CON, 13.9 ± 0.4 pA; TTX, 17.5 pA ± 0.5 pA; p = 4.92 x 10⁻⁷, Kruskal-Wallis test). Values are given as mean ± SEM.
Figure 4. Experimental data differ from a simulation of perfect uniform multiplicative scaling. (A) Cumulative distributions of mEPSC amplitudes randomly sampled with n = 30/cell from 86 control cells (total n = 2580, black line) and 77 TTX-treated cells (total n = 2310, vermillion line). (B) Uniform multiplicative scaling was simulated by multiplying the cumulative distribution of amplitudes from control cells (black line) by a scaling factor of 1.25 (vermillion line). (C) Cumulative distributions of mEPSC amplitudes sampled by computing 30 evenly-spaced quantiles from 87 control cells and 77 TTX-treated cells. Insets: expanded view of the first quartile of the data.
Figure 5. Empirical simulations of uniform, multiplicative scaling in variable data. (A) Uniform multiplicative scaling was simulated by randomly sampling 30 mEPSC amplitudes from each untreated cell twice to obtain two simulated control distributions (simCON1, solid black line & simCON2, dashed vermillion line), then multiplying the second distribution by a scaling factor of 1.25 to simulate the hypothesized effects of TTX treatment (simTTX, solid vermillion line). (B) A detection threshold of 5 pA was simulated by removing all amplitudes less than 5 pA from the simCON and simTTX data shown in (A). (C) A detection threshold of 7 pA was simulated by removing all amplitudes less than 7 pA from the simCON and simTTX data shown in (A). Insets: expanded view of the first quartile of the data, with the 5 pA and 7 pA thresholds marked by vertical blue dashed lines.
Figure 6. The scaling transformation computed by the rank order method produces a nearly perfect fit between the downscaled TTX and control data from empirical simulations with no or a moderate detection threshold, but not in data with a high detection threshold. (A) The
coefficients from the linear regression model shown in (B) were used to downscale the simulated TTX data and produced a close match between the scaled distribution and the simulated control data (N_{simCON} = 2610, N_{simTTX} = 2610; p = 0.77, K-S test). (B) Simulated control and TTX amplitudes from the simulation of uniform scaling with no detection threshold were ranked, plotted against each other (open black circles), and fit with a linear regression model (dashed orange line). (C) The coefficients from the linear regression model shown in (D) were used to downscale the simulated TTX data and produced a close match between the scaled distribution and the simulated control data (N_{simCON} = 2594, N_{simTTX} = 2594; p = 0.63, K-S test). (D) Simulated control and TTX amplitudes from the simulation of uniform scaling with a detection threshold of 5 pA were ranked, plotted against each other (open black circles), and fit with a linear regression model (dashed orange line). (E) The coefficients from the linear regression model shown in (F) were used to downscale the simulated TTX data and failed to produce a close match, apparently due to deviations confined to the small-amplitude range of the distributions (N_{simCON} = 2321, N_{simTTX} = 2321; p = 0.01, K-S test). (F) Simulated control and TTX amplitudes from the simulation of uniform scaling with a detection threshold of 7 pA were ranked, plotted against each other (open black circles), and fit with a linear regression model (dashed orange line). Insets (A, C, E): expanded view of the first quartile of the data. Insets (B, D, F): entire range of the ranked data. Blue lines in all panels denote the first, second, and third quartiles of the data. Gray line in (B, D, F) denotes the line of identity.

Figure 7. The results of the rank order method on experimental data differ from the results on the uniformly scaled simulation data. (A) The coefficients from the linear regression model shown in (B) were used to downscale the simulated TTX data and failed to produce a close match (N_{CON} = 2580, N_{TTX} = 2310; p = 6.0 e-10, K-S test). (B) Control and TTX experimental data were resampled to yield 77 quantiles/cell from control cells and 86 quantiles/cell from TTX-treated cells, so that each experimental condition contained the same sample size (n = 6622). The amplitudes were ranked, plotted against each other (open black circles), and fit with a linear regression model (dashed orange line). Inset (A): expanded view of the first quartile of the data. Inset (B): entire range of the ranked data. Blue lines in all panels denote the first, second, and third quartiles of the data. Gray line in (B) denotes the line of identity.
Figure 8. The scaling transformation computed by the iterative method produces a nearly perfect fit between the downscaled TTX and control data from the empirical simulation with a detection threshold of 7 pA. (A) Each scaling factor was used to downscale the simulated TTX distribution, subthreshold events were discarded from the downscaled data, and the scaled distribution was compared to simulated control with a K-S test to produce the corresponding p-value. (B) The scaling factor that produced the largest p-value in (A) (1.335) was used to downscale the simulated TTX distribution and subthreshold events (n = 324, 14.0% of total samples) were discarded to produce the scaled simulated TTX distribution (dashed vermillion line). The downscaled TTX distribution was a close match to the control distribution (N_{con} = 2321, N_{ttx} = 1997; p = 0.97, K-S test). (C) The same process was repeated without discarding subthreshold amplitudes: each scaling factor was used to downscale the simulated TTX data, and the p-value (K-S) resulted from comparing all downscaled TTX data to the simulated control data. (D) The scaling factor that produced the best fit (1.165) was used to downscale the simulated TTX data, and the resulting distribution (Scaled simTTX, dashed vermillion line) was plotted against the simulation’s control data (solid black line). The downscaled TTX distribution was a poor match to the control distribution (N_{con} = 2321, N_{ttx} = 2321; p = 0.0024, K-S test).
Figure 9. The scaling transformation computed by the iterative method failed to produce a close match between downscaled TTX and control distributions in experimental data. (A) Each scaling factor was used to downscale the TTX distribution, subthreshold events were discarded from the downscaled data, and the scaled distribution was compared to control with a K-S test to produce the corresponding p-value. (B) The scaling factor that produced the largest p-value in (A) was used to downscale the TTX distribution and subthreshold events (n = 2, 0.1% of total samples) were discarded to produce the scaled TTX distribution (dashed vermillion line). The downscaled TTX distribution was not a close match to the control distribution (solid black line) (N_{con} = 2580, N_{ttx} = 2308; p = 3.0 \times 10^{-5}, \text{K-S test}).
Figure 10. Bootstrap validation and estimation of the test statistic distribution confirm that the null hypothesis of uniform scaling is rejected in experimental data. (A) Bootstrap distribution of the slope coefficients produced by repeatedly applying the rank order method to two resampled groups of control cells. (B) Bootstrap distribution of the intercept coefficients produced by repeatedly applying the rank order method to two resampled groups of control cells. (C) Bootstrap distribution of the Anderson-Darling (A-D) standardized test statistics produced by repeatedly applying the rank-order method to two resampled groups of control cells and comparing the resulting scaled distributions with the A-D test. The critical value is defined as the 95th percentile of the distribution. (D) Results of the rank order method on experimental data (Figure 7). An A-D test comparing the downscaled TTX distribution to control yielded a standardized test statistic of 29.6, which exceeds the critical value of 8.37, resulting in rejection of the null hypothesis that the two distributions are equivalent. A p-value was calculated as the test statistic’s percentile in the bootstrap test statistic distribution.
Figure 11. The ratio of TTX to control amplitudes is approximately uniform in simulation data, with minor deviations caused by detection thresholds, but is non-uniform over most of the range of the experimental data. (A) Ratio of simulated TTX to simulated control in data from the simulation of uniform scaling with no detection threshold. (B) Ratio of simulated TTX to simulated control in data from the simulation of uniform scaling with a detection threshold of 5 pA. (C) Ratio of simulated TTX to simulated control in data from the simulation of uniform scaling with a detection threshold of 7 pA. (D) Ratio of TTX to control in experimental data from mouse cortical neurons. All panels: horizontal dashed vermillion lines denote the expected value of the TTX:CON ratio, defined as the slope coefficients of the linear regression model fit to the respective data. Vertical blue dashed lines denote the value of the detection threshold, where applicable. Vertical blue solid lines denote the first, second, and third quartiles of the data.
Figure 12. Distributions of mEPSC amplitudes from control and TTX-treated neurons are not fit by a normal distribution but are well fit by a generalized extreme value (GEV) distribution. (A) Frequency histogram of the control data recorded in mouse cortical neurons, fit by a normal distribution (orange) and a GEV distribution (blue). (B) Frequency histogram of the TTX-treated data recorded in mouse cortical neurons, fit by a normal distribution (orange) and a GEV distribution (blue). (C) Example normal distribution with μ (mean) = 0 and σ (standard deviation) = 1. (D) Example GEV distributions with μ = 0, σ = 1, and ξ (shape) = 0 (solid blue line), and μ = 0, σ = 1, and ξ = 0.4.
Figure 13. Step by step illustration of the comparative standardization process used to transform one sample distribution GEV1 to fit a second sample distribution GEV2. (A) A frequency histogram of GEV1 was plotted alongside GEV2 (solid blue line). (B) A cumulative distribution of GEV1 (solid black line) was plotted alongside GEV2 (solid blue line). (C) A frequency histogram of GEV1 multiplied by the multiplicative factor (1.39) calculated using
comparative standardization of GEV1 to GEV2 (solid blue line). (D) A cumulative distribution of GEV1 was multiplied by 1.39 (solid black line) and plotted alongside GEV2 (solid blue line). (E) A frequency histogram of GEV1 scaled by the multiplicative (1.39) and additive (-1.80) factors calculated using comparative standardization of GEV1 to GEV2 (solid blue line). (F) A cumulative distribution of GEV1 scaled by the multiplicative and additive factors (solid black line) and plotted alongside GEV2 (solid blue line).
Figure 14. Possible outcomes of comparative standardization. (A) CDFs of sample distribution GEV1 and artificially scaled data with a negative additive factor. (B) Ratio plot of artificially scaled data with a negative additive factor. (C) CDFs of GEV1 and artificially scaled data with an additive factor of zero. (D) Ratio plot of artificially scaled data with an additive factor of zero. (E)
CDFs of GEV1 and artificially scaled data with a positive additive factor. **F** Ratio plot of artificially scaled data with a positive additive factor.

**Figure 15.** Comparative standardization computes the expected scaling factors, which correspond to transformation by identity, when used to compare two sets of untreated data. **(A)** Bootstrap distribution of the multiplicative factors produced by repeatedly applying comparative standardization to two resampled groups of control cells. **(B)** Bootstrap distribution of the additive factors produced by repeatedly applying comparative standardization to two resampled groups of control cells. **(C)** Bootstrap distribution of the Anderson-Darling (A-D) standardized test statistics produced by repeatedly applying comparative standardization to two resampled groups of control cells and comparing the resulting scaled distributions with the A-D test. The critical value is defined as the 95th percentile of the distribution.
Figure 16. Comparative standardization computes the correct scaling factors when tested on data artificially scaled by a uniform multiplicative factor of 1.25. (A) Bootstrap distribution of the multiplicative factors produced by repeatedly applying comparative standardization to two resampled groups of control cells, of which one group was scaled by 1.25. (B) Bootstrap distribution of the additive factors produced by repeatedly applying comparative standardization to two resampled groups of control cells, of which one group was scaled by 1.25.
Figure 17. Comparative standardization computes a transformation that results in no significant difference between the TTX-treated and scaled control data recorded in mouse cortical neurons. (A) A frequency histogram of the combined control and TTX data, fit with a generalized extreme value distribution (solid blue line). (B) A frequency histogram of the control data, fit with a generalized extreme value distribution with $\xi = 0.42$. (C) A frequency
histogram of the TTX data, fit with a generalized extreme value distribution with $\xi = 0.42$. (D) Results of comparative standardization on experimental data. An A-D test comparing the scaled control distribution to TTX yielded a standardized test statistic of -0.163, which does not exceed the critical value of 0.009 (Figure 15), resulting in failure to reject the null hypothesis that the two distributions are equivalent. A p-value was calculated as the test statistic’s percentile in the bootstrap test statistic distribution.

![Bootstrap distributions of scaling factors for experimental data](image)

**Figure 18.** Bootstrap estimation of the distribution of comparative-standardization scaling factors for experimental data indicates that uniform scaling, which corresponds to an additive factor of 0, is extremely unlikely. (A) Bootstrap distribution of the multiplicative factors produced by repeatedly applying comparative standardization to a resampled group of control cells and a resampled group of TTX cells. (B) Bootstrap distribution of the additive factors produced by repeatedly applying comparative standardization to a resampled group of control cells and a resampled group of TTX cells.
Figure 19. Comparative standardization computes a transformation that results in a better fit between scaled control and TTX experimental data (mouse cortical neurons) than the transformations computed by the rank order and iterative methods. (A) The coefficients calculated using the rank order method (Figure 7) were used to scale the control data; the resulting scaled control distribution (dashed black line) was compared to the TTX distribution (solid vermillion line) with an A-D test. (B) The scaling factor calculated using the iterative method (Figure 9) was used to scale the control data; the resulting scaled control distribution (dashed black line) was compared to the TTX distribution from which subthreshold events were removed (solid vermillion line) with an A-D test. (C) The multiplicative and additive factors calculated using comparative standardization (Figure 17) were used to scale the control data; the resulting scaled control distribution (dashed black line) was compared to the TTX distribution (solid vermillion line) with an A-D test.
Figure 20. The results of the rank order method support uniform scaling in rat cortical neurons treated with CNQX, but not rat cortical neurons treated with TTX or mouse hippocampal neurons treated with TTX. (A) The coefficients from the linear regression model shown in (B)
were used to downscale the TTX data and failed to produce a close match between scaled TTX and control ($N_{\text{con}} = 1410$, $N_{\text{ttx}} = 1740$; $p = 2.9 \times 10^{-7}$, K-S test). (B) Control and TTX experimental data were resampled to yield 58 quantiles/cell from control cells and 47 quantiles/cell from TTX-treated cells, so that each experimental condition contained the same sample size ($n = 2726$). The amplitudes were ranked, plotted against each other (open black circles), and fit with a linear regression model (dashed orange line). (C) The coefficients from the linear regression model shown in (D) were used to downscale the CNQX data and produced a close match between scaled CNQX and control ($N_{\text{con}} = 2670$, $N_{\text{cnq}} = 2820$; $p = 0.13$, K-S test). (D) Control and CNQX experimental data were resampled to yield 94 quantiles/cell from control cells and 89 quantiles/cell from CNQX-treated cells, so that each experimental condition contained the same sample size ($n = 8366$). The amplitudes were ranked, plotted against each other (open black circles), and fit with a linear regression model (dashed orange line). (E) The coefficients from the linear regression model shown in (F) were used to downscale the TTX data and failed to produce a close match between scaled TTX and control ($N_{\text{con}} = 540$, $N_{\text{ttx}} = 600$; $p = 7.7 \times 10^{-3}$, K-S test). (F) Control and TTX experimental data were resampled to yield 20 quantiles/cell from control cells and 18 quantiles/cell from TTX-treated cells, so that each experimental condition contained the same sample size ($n = 360$). The amplitudes were ranked, plotted against each other (open black circles), and fit with a linear regression model (dashed orange line). Inset (A, C, E): expanded view of the first quartile of the data. Inset (B, D, F): entire range of the ranked data. Blue lines in all panels denote the first, second, and third quartiles of the data. Gray line in (B, D, F) denotes the line of identity.
Figure 21. The results of the iterative method do not support uniform scaling in rat cortical neurons treated with TTX, rat cortical neurons treated with CNQX, or mouse hippocampal neurons treated with TTX. (A) Each scaling factor was used to downscale the TTX distribution,
subthreshold events were discarded from the downscaled data, and the scaled distribution was compared to control with a K-S test to produce the corresponding p-value. **(B)** The scaling factor that produced the largest p-value in (A) was used to downscale the TTX distribution and subthreshold events (n = 25, 1.4% of total samples) were discarded to produce the scaled TTX distribution (dashed vermillion line). The downscaled TTX distribution was not a close match to the control distribution (solid black line) (N_{con} = 1410, N_{ttx} = 1715; p = 2.4 \times 10^{-4}, K-S test). **(C)** Each scaling factor was used to downscale the CNQX distribution, subthreshold events were discarded from the downscaled data, and the scaled distribution was compared to control with a K-S test to produce the corresponding p-value. **(D)** The scaling factor that produced the largest p-value in (C) was used to downscale the CNQX distribution and subthreshold events (n = 70, 2.5% of total samples) were discarded to produce the scaled CNQX distribution (dashed vermillion line). The downscaled CNQX distribution was not a close match to the control distribution (solid black line) (N_{con} = 2670, N_{cnq} = 2750; p = 6.2 \times 10^{-5}, K-S test). **(E)** Each scaling factor was used to downscale the TTX distribution, subthreshold events were discarded from the downscaled data, and the scaled distribution was compared to control with a K-S test to produce the corresponding p-value. **(F)** The scaling factor that produced the largest p-value in (E) was used to downscale the TTX distribution and subthreshold events (n = 282, 47% of total samples) were discarded to produce the scaled TTX distribution (dashed vermillion line). Although the downscaled TTX distribution appears to be a close match to the control distribution (solid black line) (N_{con} = 540, N_{ttx} = 318; p = 0.76, K-S test), the high scaling factor caused nearly half of the downscaled TTX data to be discarded so this cannot be considered a good fit.

**Figure 22.** The ratio of TTX to control amplitudes is non-uniform over most of the amplitude range in data from rat cortical neurons treated with TTX, rat cortical neurons treated with CNQX, and mouse hippocampal neurons treated with TTX. **(A)** Ratio of TTX to control amplitudes in data recorded in rat cortical neurons. **(B)** Ratio of CNQX to control amplitudes in data recorded in rat cortical neurons. **(C)** Ratio of TTX to control amplitudes in data recorded in mouse hippocampal neurons. All panels: horizontal dashed vermillion lines denote the expected value of the ratio, defined as the slope coefficients of the linear regression model fit to the respective data. Vertical blue dashed lines denote the value of the detection threshold. Vertical blue solid lines denote the first, second, and third quartiles of the data.
Figure 23. The ratio of TTX to control amplitudes varies widely with experimental conditions but is consistently non-uniform. (A) Ratio of TTX to control amplitudes in data recorded in rat cortical neurons (Figure 22A). (B) Ratio of CNQX to control amplitudes in data recorded in rat cortical neurons (Figure 22A), with expanded axes. (C) Ratio of TTX to control amplitudes in data recorded in mouse cortical neurons (Figure 11D), with expanded axes. (D) Ratio of TTX to control amplitudes in data recorded in mouse hippocampal neurons (Figure 22C). All panels: horizontal dashed vermillion lines denote the expected value of the ratio, defined as the slope coefficients of the linear regression model fit to the respective data. Vertical blue dashed lines denote the value of the detection threshold. Vertical blue solid lines denote the first, second, and third quartiles of the data.
Figure 24. Comparative standardization computes a transformation that results in a better fit between scaled control and TTX-treated data than the transformations computed by the rank order and iterative methods in rat cortical neurons. (A) Frequency histogram of the control data recorded in rat cortical neurons, fit by a GEV distribution (blue). (B) Frequency histogram of the TTX-treated data recorded in rat cortical neurons, fit by a GEV distribution (blue). (C) The coefficients calculated using the rank order method (Figure 20A-B) were used to scale the control data; the resulting scaled control distribution (dashed black line) was compared to the TTX distribution (solid vermillion line) with an A-D test. (D) The scaling factor calculated using the iterative method (Figure 21A-B) was used to scale the control data; the resulting scaled control distribution (dashed black line) was compared to the TTX distribution from which subthreshold events were removed (solid vermillion line) with an A-D test. (E) The multiplicative and additive factors calculated using comparative standardization were used to scale the control data; the resulting scaled control distribution (dashed black line) was compared to the TTX distribution (solid vermillion line) with an A-D test.
Figure 25. Comparative standardization computes a transformation that results in a better fit between scaled control and CNQX-treated data than the transformations computed by the rank order and iterative methods in rat cortical neurons. (A) Frequency histogram of the control data recorded in rat cortical neurons, fit by a GEV distribution (blue). (B) Frequency histogram of the CNQX-treated data recorded in rat cortical neurons, fit by a GEV distribution (blue). (C) The coefficients calculated using the rank order method (Figure 20C-D) were used to scale the control data; the resulting scaled control distribution (dashed black line) was compared to the TTX distribution (solid vermillion line) with an A-D test. (D) The scaling factor calculated using the iterative method (Figure 21C-D) was used to scale the control data; the resulting scaled control distribution (dashed black line) was compared to the TTX distribution from which subthreshold events were removed (solid vermillion line) with an A-D test. (E) The multiplicative and additive factors calculated using comparative standardization were used to scale the control data; the resulting scaled control distribution (dashed black line) was compared to the TTX distribution (solid vermillion line) with an A-D test.
Figure 26. Comparative standardization computes a transformation that results in a better fit between scaled control and TTX-treated data than the transformations computed by the rank order and iterative methods in mouse hippocampal neurons. **(A)** Frequency histogram of the control data recorded in mouse hippocampal neurons, fit by a GEV distribution (blue). **(B)** Frequency histogram of the TTX-treated data recorded in mouse hippocampal neurons, fit by a GEV distribution (blue). **(C)** The coefficients calculated using the rank order method (Figure 20E-F) were used to scale the control data; the resulting scaled control distribution (dashed black line) was compared to the TTX distribution (solid vermillion line) with an A-D test. **(D)** The scaling factor calculated using the iterative method (Figure 21E-F) was used to scale the control data; the resulting scaled control distribution (dashed black line) was compared to the TTX distribution from which subthreshold events were removed (solid vermillion line) with an A-D test. **(E)** The multiplicative and additive factors calculated using comparative standardization were used to scale the control data; the resulting scaled control distribution (dashed black line) was compared to the TTX distribution (solid vermillion line) with an A-D test.
Figure 27. The multiplicative and additive factors computed by comparative standardization are strongly negatively correlated. The additive factors calculating using comparative standardization were plotted as a function of the multiplicative factor on the same data.

Comparison of scaling factors computed by the rank order method and comparative standardization

Figure 28. Lack of correlation between the scaling factors from the rank order method and comparative standardization suggests that comparative standardization detects a different transformation than the rank order method, rather than a more accurate or precise version of the same transformation. (A) The multiplicative factors calculated using comparative standardization, plotted as a function of the slope coefficients calculated using the rank order method on the same data. (B) The additive factors calculated using comparative standardization, plotted as a function of the slope coefficients calculated using the rank order method on the same data.
Figure 29. Coefficients computed by the rank order method correlate weakly with detection threshold, indicating that the intercept coefficient is unlikely to be an effective correction for the effects of a threshold. (A) Slope coefficients calculated using the rank order method on experimental data, as a function of the detection threshold in the respective data. (B) Intercept coefficients calculated using the rank order method on experimental data, as a function of the detection threshold in the respective data.
Figure 30. A detection threshold causes comparative standardization to overestimate the magnitude of the homeostatic plasticity effect, but the overestimation is small relative to the total magnitude of the factors. (A) Multiplicative vs. additive factors calculated using comparative standardization on experimental and simulation data. (B) Multiplicative factors calculated using comparative standardization on experimental and simulation data, as a function of the detection threshold for the respective data. (C) Additive factors calculated using comparative standardization on experimental and simulation data, as a function of the detection threshold for the respective data.
**Figure 31.** Hypothesized appearance of homeostatic divergent downscaling of mEPSC amplitudes in response to increased firing activity. (A) CDFs showing a simulated divergent-scaling increase in mEPSC amplitudes. (B) Ratio plot of a simulated divergent-scaling increase in mEPSC amplitudes. (C) CDFs showing a simulated divergent-scaling decrease in mEPSC amplitudes. (D) Ratio plot of a simulated divergent-scaling decrease in mEPSC amplitudes.
### VIII. TABLES

**Table 1.** Summary of experimental data sets

<table>
<thead>
<tr>
<th>Species</th>
<th>Tissue</th>
<th>Animal age</th>
<th>Days in vitro</th>
<th>Treatment</th>
<th>Detection threshold</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Cortex</td>
<td>P0</td>
<td>13-14</td>
<td>TTX</td>
<td>3 pA</td>
<td>(Koesters 2015)</td>
</tr>
<tr>
<td>Rat</td>
<td>Cortex</td>
<td>E18</td>
<td>8-12</td>
<td>TTX</td>
<td>5 pA</td>
<td>(Fong et al. 2015)</td>
</tr>
<tr>
<td>Rat</td>
<td>Cortex</td>
<td>E18</td>
<td>8-12</td>
<td>CNQX</td>
<td>5 pA</td>
<td>(Fong et al. 2015)</td>
</tr>
<tr>
<td>Mouse</td>
<td>Hippocampus</td>
<td>P0-1</td>
<td>12-15</td>
<td>TTX</td>
<td>7 pA</td>
<td>(Altimimi and Stellwagen 2013)</td>
</tr>
</tbody>
</table>

**Table 2.** Results of the rank-order, iterative, and comparative standardization methods on experimental data sets.

<table>
<thead>
<tr>
<th>Data set</th>
<th>Rank-order</th>
<th>Comparative standardization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>slope</td>
<td>intercept</td>
</tr>
<tr>
<td>Mouse cortical, TTX</td>
<td>1.23</td>
<td>0.4</td>
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<tr>
<td>Rat cortical, TTX</td>
<td>2.29</td>
<td>-10.52</td>
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<tr>
<td>Rat cortical, CNQX</td>
<td>1.65</td>
<td>-2.74</td>
</tr>
<tr>
<td>Mouse hippocampal, TTX</td>
<td>1.89</td>
<td>-4.49</td>
</tr>
</tbody>
</table>
IX. EQUATIONS

\[
\frac{x - \mu_x}{\sigma_x} = x_{std}
\]

**Equation 1.** Formula for data standardization. A sample \(x\) can be standardized, or transformed to have a mean of 0 and standard deviation of 1, by subtracting the mean and dividing by the standard deviation for every value in \(x\).

\[
GEV(0,1,\xi_1) = GEV(0,1,\xi_2) \text{ if } \xi_1 = \xi_2
\]

**Equation 2.** Two generalized extreme value (GEV) distributions with location parameter \(\mu = 0\) and scale parameter \(\sigma = 1\) are equivalent if and only if their shape parameters \(\xi\) are equal.
\[
\frac{GEV_1 - \mu_1}{\sigma_1} = GEV(0, 1, \xi) \quad (a)
\]

\[
\frac{GEV_2 - \mu_2}{\sigma_2} = GEV(0, 1, \xi) \quad (b)
\]

\[
\frac{GEV_1 - \mu_1}{\sigma_1} = \frac{GEV_2 - \mu_2}{\sigma_2} \quad (c)
\]

\[
\frac{\sigma_2}{\sigma_1} (GEV_1 - \mu_1) = GEV_2 - \mu_2
\]

\[
\frac{\sigma_2}{\sigma_1} (GEV_1 - \mu_1) + \mu_2 = GEV_2
\]

\[
\frac{\sigma_2}{\sigma_1} GEV_1 + \mu_2 - \frac{\sigma_2}{\sigma_1} \mu_1 = GEV_2
\]

\[
GEV_1 \times f_{\text{mult}} + f_{\text{add}} = GEV_2 \quad (d)
\]

\[
f_{\text{mult}} = \frac{\sigma_2}{\sigma_1}
\]

\[
f_{\text{add}} = \mu_2 - \frac{\sigma_2}{\sigma_1} \mu_1
\]

**Equation 3.** Derivation of the comparative standardization formula. *(a)* Data that are fit by an arbitrary generalized extreme value distribution, GEV\(_1\), can be transformed to fit a generalized extreme value distribution with \(\mu = 0\) and \(\sigma = 1\) by subtracting \(\mu_1\) and dividing by \(\sigma_1\). *(b)* Data that are fit by a second arbitrary generalized extreme value distribution, GEV\(_2\), can be transformed to fit a generalized extreme value distribution with \(\mu = 0\) and \(\sigma = 1\) by subtracting \(\mu_2\) and dividing by \(\sigma_2\). *(c)* Per Equation 2, if GEV\(_1\) and GEV\(_2\) have \(\xi\) parameters with the same value, their transformed version can be set equal and solved algebraically for GEV\(_2\), yielding the equation to transform GEV\(_1\) to fit GEV\(_2\). *(d)* The transformation equation can then be defined in terms of a multiplicative factor \(f_{\text{mult}}\) and additive factor \(f_{\text{add}}\).
$GEV_{con} \times f_{mult} + f_{add} = GEV_{ttx}$

$f_{mult} = \frac{\sigma_{ttx}}{\sigma_{con}}$

$f_{add} = \mu_{ttx} - \frac{\sigma_{ttx}}{\sigma_{con}} \mu_{con}$

**Equation 4.** The comparative standardization formula used to transform a distribution of mEPSC amplitudes recorded in untreated neurons to match a distribution of amplitudes recorded in neurons treated with TTX to induce homeostatic plasticity.
X. BIBLIOGRAPHY


Scudder, S. L., M. S. Goo, A. E. Cartier, A. Molteni, L. A. Schwarz, R. Wright, and G. N. Patrick. 2014. 'Synaptic strength is bidirectionally controlled by opposing activity-dependent regulation of Nedd4-1 and USP8', *J Neurosci*, 34: 16637-49.


