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Neurochemistry

Single-Vesicle Electrochemistry Following Repetitive Stimulation Reveals a Mechanism for Plasticity Changes with Iron Deficiency

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Abstract: Deficiency of iron, the most abundant transition metal in the brain and important for neuronal activity, is known to affect synaptic plasticity, causing learning and memory deficits. How iron deficiency impacts plasticity by altering neurotransmission at the cellular level is not fully understood. We used electrochemical methods to study the effect of iron deficiency on plasticity with repetitive stimulation. We show that during iron deficiency, repetitive stimulation causes significant decrease in exocytotic release without changing vesicular content. This results in a lower fraction of release, opposite to the control group, upon repetitive stimulation. These changes were partially reversible by iron repletion. This finding suggests that iron deficiency has a negative effect on plasticity by decreasing the fraction of vesicular release in response to repetitive stimulation. This provides a putative mechanism for how iron deficiency modulates plasticity.

Introduction

Iron, the most abundant transition metal in the brain, is indispensable for maintaining normal brain functions. Iron acts as a protein cofactor participating in numerous cellular and neurodevelopmental processes, including energy production, neurotransmitter synthesis, and synaptic development.[1] The brain is particularly sensitive to the disruption of iron homeostasis. Such modifications, either iron overload or iron deficiency, can be detrimental and induce both disruption of neurophysiological mechanism and severe neuropathological changes. Iron overload is prominent in brain regions associated with motor dysfunction and cognitive impairments in aging populations and patients with various neurodegenerative diseases, including Parkinson's and Alzheimer's diseases.[1a] Iron deficiency is the most common nutritional disorder worldwide and affects all age groups that leads to learning and memory impairments,[2] and these deficits persist following iron repletion. The hippocampus, a region in the brain responsible for learning and memory formation, is highly susceptible to the effects of iron deficiency. During early life, iron deficiency-induced impairments in synaptic transmission and plasticity are strongly associated with deterioration of hippocampal neuronal functions. However, how iron deficiency affects learning and memory via the alterations of neurotransmission via exocytosis and plasticity on the cellular level is not fully understood.

Synaptic plasticity is widely considered to be the key in the cellular basis for learning and memory.[3] It refers to the activity-dependent neuronal response that entails significant modifications of the strength or efficacy of synaptic transmission.[4] Synaptic transmission can either be facilitated or depressed by activity, and these alterations could last from milliseconds to hours, days or even longer. Synaptic strength is tightly regulated by the changes of exocytosis at the presynaptic terminal,[5] where neurotransmitter release is mediated by small synaptic vesicles and large dense-core vesicles. Exocytosis is typically triggered by a stimulus which depolarizes the cell membrane and opens the calcium channels, resulting in an increase of intracellular calcium levels. This subsequently triggers the fusion between vesicle and cell membranes, and then the release of vesicular neurotransmitter content into the extracellular space. Recent reports have argued that regular exocytosis is predominantly via a partial release mechanism, which refers to a situation where only a fraction of the vesicular transmitter content is released and is suggested as the main mechanism of transmitter release during regular exocytosis.[6] This is thought to be critical in the elucidation of the regulation of individual vesicular release events and the understanding of synaptic strength at the single vesicle level. Changes in plasticity can be studied using a variety of methods, including but not limited to functional magnetic resonance imaging for measuring blood-oxygen-level dependent signal changes,[7] patch-clamp technique for recording intracellular current or voltage of the cell membrane,[8] and electroencephalography for recording electrical signal of the brain to study long-term potentiation like phenomena.[9]

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Molecular initiation of short-term plasticity in exocytosis has recently been examined using the paradigm of repetitive stimulation to reveal the possible correlation between plasticity and the fraction of neurotransmitter release.[10] As the effect of iron deficiency on exocytosis and vesicular neurotransmitter storage at the single cell level is unknown, there is an urgent need to understand the role of iron deficiency on exocytosis to address current demands in understanding the mechanism of plasticity as well as learning and memory deficits.

In this study, we investigated the effect of iron deficiency on plasticity via the paradigm of repetitive stimulation. To examine the relationship between iron deficiency and exocytotic plasticity at the cellular level, PC12 cells, a major model for neurosecretion and neural differentiation studies, were used to model iron deficiency and iron repletion.[11] Electrochemical methods, single cell amperometry (SCA) and intracellular vesicle impact electrochemical cytometry (IVIEC), were used to study the effects of iron deficiency on exocytotic release and vesicular content of catecholamine, respectively, in response to repetitive stimulation. Under the condition of iron deficiency, repetitive stimulation induced a significant decrease in exocytosis, whereas vesicular content was left unchanged, resulting in a lower fraction of release, a trend opposite to that of control cells. Interestingly, these changes are only partially rescued by subsequent iron repletion. These results suggest that alteration in plasticity can be observed as changes in fraction of release following repetitive stimulation, and the alteration in fraction of release induced by disruption of iron homeostasis is likely to be one of the mechanisms that leads to negative impact on plasticity and causes long-term deficits in learning and memory.

Results and Discussion

To investigate the effect of iron on exocytosis of catecholamine, the iron chelator deferoxamine mesylate (DFOM), which is widely applied in iron chelation studies, was used to induce iron deficiency in PC12 cells.[12] For DFOM treatment, cells were incubated in 100 μM DFOM supplemented medium for 24 h. For iron repletion, cells were treated with 100 μM DFOM for 24 h and subsequently followed by 24 h rescue with treatment of 100 μM ferric ammonium citrate (FAC). The concentrations of DFOM and FAC were chosen based on previous studies of iron deficiency and iron chelation experiments in PC12 cells.[13] Electrochemical techniques, SCA and IVIEC, were used to study the effects of iron deficiency on exocytotic release and vesicular content of catecholamine. The large dense-core secretory vesicles in PC12 cells contain catecholamine transmitters, among which are mainly dopamine and a small fraction of norepinephrine (the norepinephrine/dopamine ratio varies from 0.003 to 0.53), with no detectable level of epinephrine.[14] In SCA and IVIEC, the constant potential of +700 mV versus an Ag/AgCl reference electrode is sufficient to oxidize dopamine and norepinephrine at the electrode. SCA was used to quantify the amount of transmitter release during single vesicle exocytosis and monitor the release dynamics of individual exocytotic events. In SCA, a carbon fiber disk microelectrode is placed above a single PC12 cell. The cell is stimulated with 100 mM K^+ solution for 5 s to evoke catecholamine release and exocytotic events are measured as amperometric spikes. Each amperometric current spike recorded during exocytosis represents a single secretory event. IVIEC was used to directly quantify the transmitter storage in individual vesicles inside a single cell. In IVIEC, a flamed-etched carbon fiber nanotip electrode is used to penetrate the cell membrane into the cytoplasm. Intracellular vesicles then adsorb and rupture on the surface of the electrode, and the total vesicular content is detected as amperometric spikes. The number of catecholamine molecules is quantified with Faraday's law ($N = Q/nF$). Previous studies have tested the limiting currents of nanotip electrodes at different insertion depths inside a single cell or synapses.[6f,15] The baseline and noise levels did not change when the whole active electrode area of the electrode was inserted into the cell and the limiting currents were restored to their original values after withdrawing the electrode from the cell or synapse, suggesting that the electrochemically active surface and sensitivity of the electrode were not affected during the insertion process. The electrochemical performance of the carbon fiber disk electrodes and nanotip electrodes were characterized using cyclic voltammetry. Electrodes that showed good response to dopamine and stable steady-state currents were used for the experiments.

Figures 1A–F show representative amperometric traces for exocytotic catecholamine release obtained by SCA (Figure 1A–C) and vesicular content of catecholamine obtained by IVIEC (Figure 1D–F) from control, DFOM treated (iron-deficient) and DFOM + FAC treated (iron-replete) cells. The average number of catecholamine molecules released per exocytotic events as well as the total vesicular catecholamine content are significantly increased in iron-deficient cells (Figure 1C and F insets, SCA: 128000 ± 9300 and IVIEC: 211000 ± 17200) compared to the control cells (SCA: 96900 ± 8500 and IVIEC: 169000 ± 11500). Interestingly, these increases by iron deficiency with DFOM are partially reversed after iron repletion with FAC, where the average numbers of released molecules and vesicular content in iron-replete cells return close to the control level (SCA: 102000 ± 8000 and IVIEC: 184000 ± 12600). Thus, iron deficiency alters exocytotic release and vesicular storage of catecholamine, and these effects seem to be partially reversible after iron is repleted.

Alteration of iron levels in the brain is associated with deficits in memory formation, and changes in neural plasticity are correlated with cognitive alteration.[16] To assess how iron deficiency affects plasticity, short interval repetitive stimulation was used to induce plasticity.[10] Simultaneously, exocytotic release and total vesicular content of catecholamine molecules were measured by SCA and IVIEC, respectively. PC12 cells were stimulated repeatedly by a 5 s 100 mM K^+ stimulation solution every 2 min for a total of 4 stimulation. This number of stimulations was chosen based on previous work, where

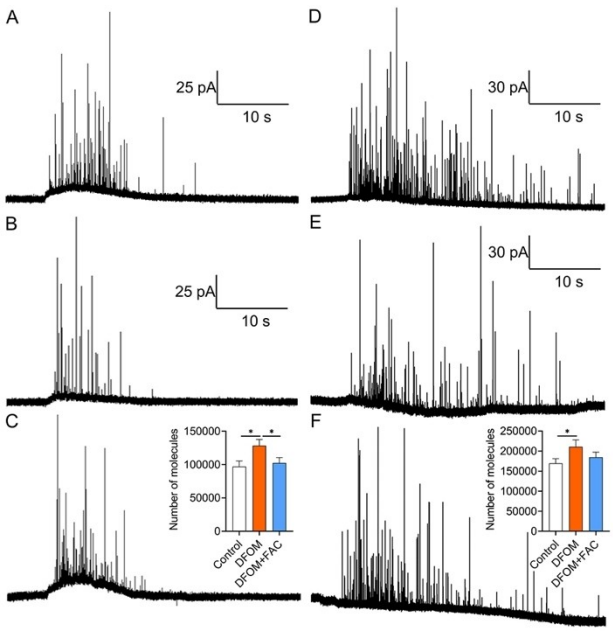

Figure 1. Representative SCA amperometric traces from A) a control, B) a DFOM treated, and C) a DFOM + FAC treated cell. Inset of C): comparison of the average number of molecules released per exocytotic event. Representative IVIEC amperometric traces from D) a control cell, E) a DFOM treated cell, and F) a DFOM + FAC treated cell. Inset of F): comparison of the average number of molecules stored in vesicles. Pairs of data sets were compared with a Mann–Whitney rank-sum test. $n > 18$ cells. p values are listed in Table S1. In IVIEC and SCA, low drift baselines are observed with carbon fiber electrodes. This baseline drift is possibly associated with the changes at the electrode surface, including non-specific adsorption of proteins and adsorption of biomolecules or byproducts of electrochemical reactions, as well as the changes occurring in extracellular or intracellular pH.

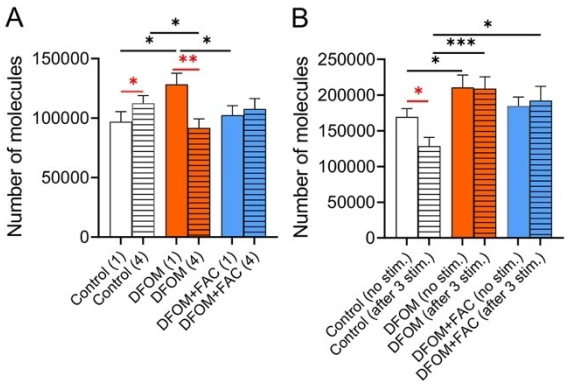

Figure 2. Effect of iron on the average amount of exocytotic release and vesicular content during repetitive stimulation. A) Average number of molecules released obtained by SCA for the 1st and the 4th stimulation from control, DFOM, and DFOM + FAC treated cells. B) Average number of molecules stored in single vesicles without stimulation and after 3 stimulations obtained by IVIEC. SCA data sets were compared with a Wilcoxon matched-pairs signed rank test within the same groups. SCA data sets between different groups and all IVIEC data sets were compared with a Mann-Whitney rank-sum test. $n > 18$ cells. p values are listed in Tables S2 and S3.

significant changes in exocytotic release, vesicular content, as well as fraction of release were observed.[10] Figure 2A shows the average number of exocytotic released catecholamine molecules during the 1st and the 4th stimulation. The number of molecules released during exocytosis increases significantly at the 4th stimulation in control cells. In contrast, a significant decrease is observed in iron-deficient cells and a slight but not significant increase is found in iron-replete cells. While repetitive stimulation increases the number of exocytotic released molecules in control cells, the opposite effect is observed in iron-deficient cells and this effect is only partially reversible when iron is replenished. The average numbers of exocytotically released molecules during each stimulation for control, iron-deficient, and iron-replete cells are shown in Figure S1. In addition, the average total vesicular content per vesicle without any stimulation and after 3 stimulations were measured by IVIEC to compare the vesicular content before the 1st to that before the 4th stimulation-induced exocytosis (Figure 2B). The average vesicular content is significantly decreased after 3 repetitive stimulations in control cells; however, it remains unaffected in iron-deficient as well as iron-replete cells by 3 repetitive stimulations, as quantified by IVIEC.

Iron deficiency-induced neurochemical changes are thought to cause cognitive impairment. In this study, iron deficiency induces significant increases in vesicular storage of catecholamine molecules before any stimulation and after 3 repetitive stimulations, compared with control (Figure 2B). The increase in the number of molecules stored in vesicles after iron depletion can be attributed to the ability of iron to alter the enzymatic activity related to catecholamine synthesis. For example, decreased iron level should result in a decreased activity of tyrosine hydroxylase (TH), as iron is an essential cofactor for the catalytic activity of TH. However, elevated levels of TH and phosphorylated TH (pTH) have been seen in previous studies of iron deficiency in both a rat model and PC12 cells.[13a] TH is known as the rate-limiting enzyme for catecholamine synthesis, where increase in TH levels likely leads to an increase in catecholamine synthesis and thus, greater amount of catecholamine molecules can be loaded in vesicles. In addition, iron depletion can alter the availability of dopamine transporters and indirectly affect enzymatic activity. Activation of dopamine D_2 receptors has long been known to inhibit dopamine synthesis,[17] where quantal size was decreased by inhibiting TH activity and reducing the availability of cytosolic dopamine for vesicular loading.[18] D_2 receptor level was robustly decreased in iron-deficient rats and PC12 cells.[13b] This decreased D_2 receptor protein level is expected to increase TH activity and the amount of cytosolic dopamine and consequently, enhance the vesicular loading of dopamine molecules.[19] Even though repetitive stimulation was speculated to deplete vesicular storage in control cells,[10] the change in TH activity induced by iron deficiency remains the vesicular content of catecholamine unchanged after repetitive stimulation. However, reduced availability of D_2 receptors and decreased levels of the dopamine transporter are not permanent effects of iron deficiency, and

these changes can be restored when iron is repleted.[13b,20] Importantly, evidence in iron deficiency studies has shown that an iron-sufficient diet for 35 days after iron deficiency in post-weaning rats was unable to reverse the early effect of iron deficiency on either TH or pTH level.[13a] This is in line with our finding that repletion of iron does not completely reverse the effect of iron deficiency on vesicular content without any stimulation as well as after repetitive stimulation, resulting in partially irreversible changes in vesicular storage of catecholamine molecules.

Repetitive stimulation induces a decrease in vesicular content (after 3 stimulations/before the 4th stimulation) but an increase in exocytotic release (at the 4th stimulation) in control cells. A more stable fusion pore has been suggested to be induced by repetitive stimulation to enhance the amount of release, even though vesicular storage is depleted.[19] However, vesicular content was unchanged but a decrease in exocytotic release was observed in iron-deficient cells. A greater amount of vesicular loading induced by iron deficiency is expected to cause more release of catecholamine during exocytosis, but this does not happen after the iron-deficient cells are repetitively stimulated. To understand the mechanisms underlying this observation, several parameters of the amperometric spikes obtained by SCA were analysed to determine the dynamics of exocytic release events (Figure 3A). Repetitive stimulation induced a significant decrease in peak current, I_{max}, during iron depletion. In addition, the I_{max} during the 4th stimulation from iron-deficient cells was also significantly lower compared to the I_{max} during the 4th stimulation from the control cells (Figure 3B), indicating less catecholamine flux through the open fusion pore. The peak half time, t_{half}, represents the duration of fusion pore during exocytosis. The value of t_{half} was significantly increased in control cells (Figure 3C) but decreased in iron-deficient cells following repetitive stimulation, suggesting the fusion pore to be less stable in the condition of iron deficiency upon repetitive stimulation. However, no obvious changes were observed following repetitive stimulation when iron is repleted. The rise time, t_{rise}, and fall time, t_{fall}, correspond to the duration of opening and closing processes of the fusion pore, respectively. As shown in Figures 3D and E, similar trends were observed in t_{rise} for iron-deficient and iron-replete cells in comparison to control cells, but t_{fall} showed the opposite effect, which suggests that repetitive stimulation slightly speed up the closing of fusion pore in iron-deficient and iron-repleted cells.

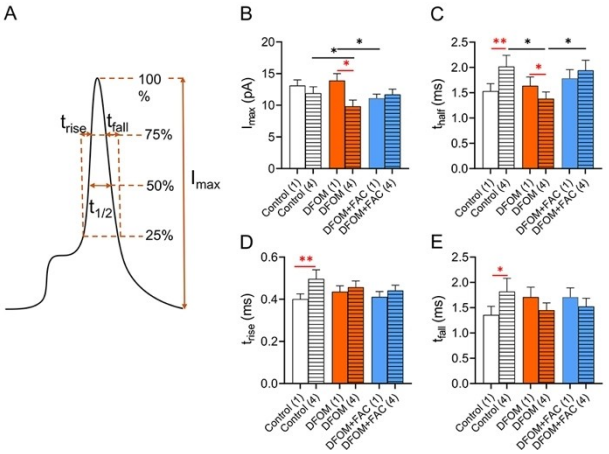

Figure 3. Amperometric peak analysis. A) Schematic of amperometric spike with different parameters. Comparisons of B) peak current, C) half peak width, D) rise time, E) fall time from SCA between the 1st and the 4th stimulation from control, DFOM, and DFOM + FAC treated cells. Data sets were compared with a Wilcoxon matched-pairs signed rank test within the same groups, and data sets between different groups were compared with a Mann-Whitney rank-sum test. $n > 18$ cells. p values are listed in Tables S4–S7.

There are slight but not significant increases in t_{half}, t_{rise} and t_{fall} in iron-deficient cells during the 1st stimulation, compared to the control. These changes may suggest that for iron deficient cells, a slightly more stable fusion pore is formed during exocytosis and additionally, the time of fusion pore opening and closing was slightly longer, allowing more catecholamine to be released during exocytosis. Regulation of exocytotic fusion pore involves many factors, including protein kinase C (PKC) and the actin cytoskeleton.[21] Iron deficiency leads to activation of PKC,[22] where decreased actin network density and actin polymerization are hallmarks in response to PKC activation.[23] PKC-mediated reduction of actin dynamics and polymerization also enhance the stability of fusion pore, which consequently increases the amount of molecules released.[21a] In addition, iron deficiency also alters dopamine function by decreasing the level/activity of dopamine D_2 receptors,[24] which modulate adenylyl cyclase activity and cAMP synthesis.[25] A decrease in the availability of D_2 receptors is likely to cause an increase in intracellular cAMP.[26] cAMP has been implicated in regulation of exocytosis by stabilizing the fusion pore and prolonging its opening.[27] This thus leads to the possible explanation for the enhancement of fusion pore stability with iron deficiency, resulting in a greater amounts of molecules released during exocytosis. However, opposite trends in t_{half} and t_{fall} were observed for iron-deficient cells following repetitive stimulation compared to the control, suggesting that the fusion pore becomes less stable after repetitive stimulation under the condition of iron deficiency. In previous studies, iron deficiency has been shown to reduce the expression level of certain genes that regulate actin polymerization, including cofilin.[28] Cofilin is a ubiquitous actin-binding protein that induces actin depolymerization. It is phosphorylated in response to theta-burst stimulation,[29] which promotes actin polymerization. The role of actin in constricting the exocytotic fusion pore has previously been suggested.[21a] In this case, it is possible that cofilin is phosphorylated upon repetitive stimulation and induces actin polymerization to constrain the fusion pore. However, cofilin is unlikely to affect the overall dynamics of fusion pore as the gene expression level of cofilin was decreased by iron deficiency, so only the total duration of the fusion pore, t_{half}, and the closure time of the fusion pore, t_{fall}, tend to become shorter upon repetitive stimulation, not the opening time of the fusion pore, t_{rise}. Subsequent iron repletion did not completely reverse the iron deficiency-

induced changes on the exocytotic dynamics, which implies that the changes in fusion pore stability caused by iron deficiency is also partially irreversible.

Modulation of exocytosis can be attributable to either the effect of quantal size or the number of exocytotic events. A significant decrease in the average number of exocytotic events is observed in iron-deficient cells at the 1st stimulation compared to control (Figure 4). Iron repletion after iron deficiency significantly elevates the number of exocytotic events, but this treatment is not sufficient to rescue the number of events back to the control level. Thus, the effect of iron deficiency on the number of exocytotic events can be persistent and last even after the replenishment of iron. In addition, with consecutive 4 repetitive stimulations, all three groups show a similar pattern of significant reduction in the number of exocytotic events (Figure 4), which can be explained as an activity-induced depletion of the pool of releasable vesicles.[10,30] The average numbers of exocytotic events gradually decrease upon repetitive stimulation for control, iron-deficient, and iron-replete cells, as shown in Figure S2. As a rapid increase in intracellular calcium level is critical to trigger exocytosis, changes in intracellular calcium level were measured by calcium imaging experiment to investigate the effect of iron deficiency on intracellular calcium dynamics. Since the patterns of changes in number of exocytotic events are similar for all three groups with 4 repetitive stimulations, only intracellular calcium changes at the 1st stimulation were measured. Stimulation of PC12 cells with 100 mM K^+ stimulation solution elicited an increase in intracellular calcium level in control cells (Figure S3). A lower number of exocytotic events is expected to be caused by, but not limited to, a lower level of calcium influx. However, the degree of calcium influx is enhanced with the treatment of DFOM and appears to be even higher when iron is replenished with FAC treatment (Figure S3), which is not in agreement with what has been observed for the number of exocytotic events (Figure 4). An alternative explanation for this finding is that the total number of vesicles in the iron-deficient or the iron-replete cells is likely reduced. To test this possibility, the average number of IVIEC events without any stimulation measured from the iron-deficient or the iron-replete cells was normalized to the control group, which was then used to estimate and compare the number of intracellular vesicles among the three groups. Significant reductions of numbers of IVIEC events before any stimulation were observed in iron-deficient cells and iron-replete cells compared to the control cells (Figure S4), where the number of vesicles decreased to 45 % in iron-deficient cells and partially reversed to 66 % in iron-deplete cells. This indicates that the number of intracellular vesicles decreases in iron deficient cells. Iron repletion partially reversed the effect of iron deficiency that brings the number of intracellular vesicles slightly back to the control level, which agrees with the observation in Figure 4. However, it should be noted that the variances in the shape of the nanotip electrodes and the depth of electrode insertion in IVIEC can make it difficult to carry out quantitative measurements and comparisons regarding the total number of intracellular vesicles, so the results shown in Figure S4 can only be considered as a rough estimation. Taken together, these results imply that iron deficiency results in a decreased total number of intracellular vesicles and fewer releasable vesicles, and these even further decreased after repetitive stimulation.

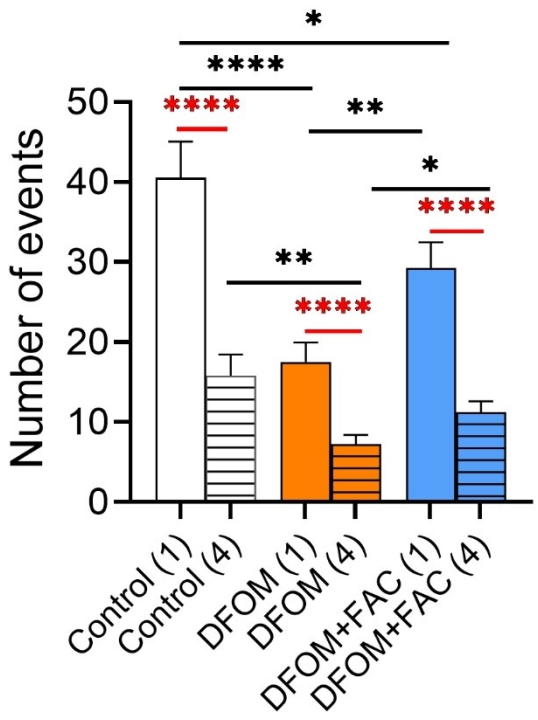

Figure 4. Average number of exocytotic events per cell. Comparison of the number of exocytotic events between the 1st and the 4th repetitive stimulation from control, DFOM treated, and DFOM + FAC treated cells. Data sets were compared with a Wilcoxon matched-pairs signed rank test within the same groups, and data sets between different groups were compared with a Mann–Whitney rank-sum test. $n > 18$ cells. p values are listed in Table S8.

The synapse is a highly specialized junction between a presynaptic and a postsynaptic cell, which allows transferring and processing of information in the central nervous system. Modulation of exocytosis is an attractive target to regulate synaptic strength and synaptic communication can be altered through a range of outputs by exocytosis.[31] Evidence has revealed that partial or sub-quantal release of vesicular content is the primary release mechanism of exocytosis in many cell types.[6] In partial release, only a fraction of vesicular content is released into the extracellular space, and vesicles can then be directly refilled with transmitters for reuse. To gain further insights into the exocytotic changes that occur under iron treatments and upon repetitive stimulation, the fraction of catecholamine release was calculated for the 1st and the 4 repetitive stimulations. The fraction of release is calculated by dividing the number of exocytotic released molecules during a stimulation over the total number of molecules stored inside individual vesicles before the stimulation. As shown in Figure 5, 56 ± 2 % of catecholamine is released during the 1st stimulation in control cells, and the fraction of release increased to 87 ± 3 % after 4 repetitive stimulations. However, the change of

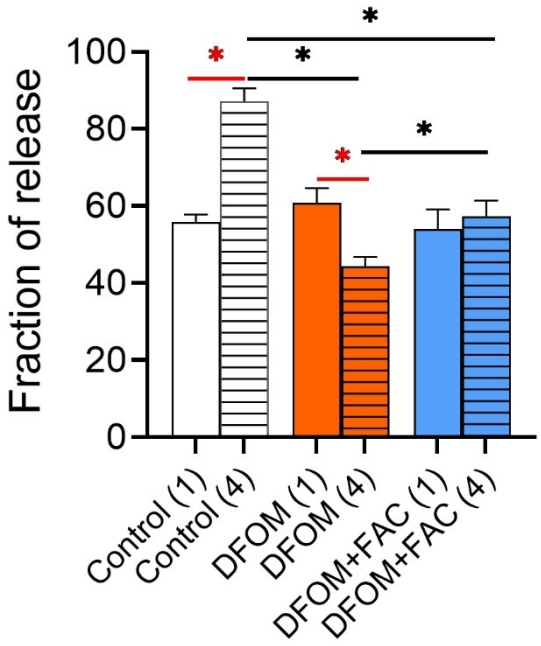

Figure 5. Fraction of release. Fraction of release was calculated for the 1st and the 4th repetitive stimulation for control, DFOM, and DFOM + FAC treated cells. Data sets were compared with a Wilcoxon matched-pairs signed rank test within the same groups, and data sets between different groups were compared with a Mann–Whitney rank-sum test. $n > 18$ cells. p values are listed in Table S9.

fraction of release in response to repetitive stimulation is in the opposite direction when iron is deficient, where vesicles release a smaller fraction of their vesicular content, from 61 ± 4 % (the 1st stimulation) to 44 ± 2 % (the 4th stimulation). This suggests that for the 1st stimulation, the induction of a relatively more stable fusion pore caused by iron deficiency is more probable than the enhancement of catecholamine synthesis, leading to a slightly higher fraction of release compared to control. While repetitive stimulation under the condition of iron deficiency leads to a less stable fusion pore and therefore, the fraction of release is likely to be smaller. Importantly, the iron deficiency-induced changes in fraction of release are not reversible with iron repletion, where the fraction of release for the 1st and the 4th repetitive stimulation remain almost the same, 54 ± 5 % and 57 ± 4 %, respectively. Thus, despite iron repletion after the condition of iron deficiency, there is a persistent dysregulation of exocytosis, as the fraction of release after repetitive stimulation does not return to the control level. A positive correlation between fraction of release and plasticity has been proposed.[10] Here, we show that repetitive stimulation causes the fraction of release to decrease during iron deficiency. Considering the negative effect of iron deficiency on plasticity, this further supports a connection between fraction of release and plasticity. We applied this theory to gain insight regarding whether and to what extent iron supplementation after iron deficiency affects the recovery of plasticity, and the unchanged fraction of release observed following repetitive stimulation indicates a possible but limited degree of plasticity recovery caused by iron repletion after iron deficiency.

A proposed mechanism for how iron deficiency affects neurotransmission (without repetitive stimulation) and how it causes alteration in plasticity by decreasing the fraction of release during exocytosis in response to repetitive stimulation is depicted in Figure 6. Repetitive stimulation of iron deficient cells leads to a decrease in the number of exocytotic events, which is probably due to activity-induced exhausting of the pool of releasable vesicles, a phenomenon also observed in the control cells.[10] Additionally, the amount of exocytotic released catecholamines is decreased upon repetitive stimulation but the vesicular catecholamine storage remains unchanged, resulting in a smaller fraction of release. Several factors can be responsible for the alteration of the fraction of release induced by iron deficiency. For example, increased TH level enhances catecholamine synthesis, so that more catecholamine molecules are available to be loaded into vesicles and subsequently released during exocytosis. Iron deficiency also causes activation of PKC to

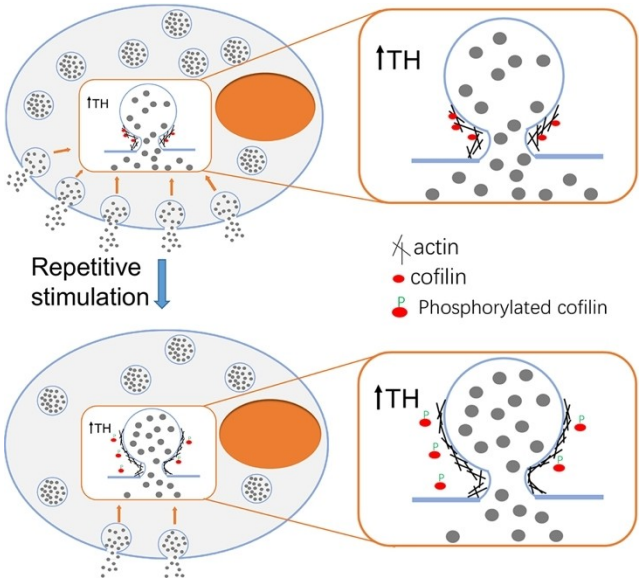

Figure 6. Proposed scheme for the effect of iron deficiency on exocytosis (without repetitive stimulation) and the effect of repetitive stimulation on exocytotic release and vesicular content with iron deficiency. Iron deficiency alone (without repetitive stimulation) gives rise to an increase in both exocytotic release and vesicular content, a slightly more stabilized fusion pore, and slightly higher fraction of release. In iron-deficient cells, repetitive stimulation leads to a decrease in the number of vesicles that fuse with the plasma membrane to release their contents into the extracellular space. Repetitive stimulation also decreases the amount of exocytotic catecholamine release without changing the vesicular catecholamine storage, resulting in a smaller fraction of release. Several factors can be responsible for the slight alteration of the fraction of release induced by iron deficiency, including enhancement of catecholamine synthesis by increased tyrosine hydroxylase (TH) levels and relatively increased stabilization of the exocytotic fusion pore by activations of protein kinase C (PKC) and cofilin. In addition, phosphorylation of cofilin upon repetitive stimulation induces a less stable fusion pore with a slightly faster closure time, resulting in a smaller fraction of release.

decrease actin network density and actin polymerization,[22] inducing a slightly more stabilized exocytotic fusion pore. Enhancement of the intracellular calcium level can activate calcineurin and subsequently induces activation of cofilin to depolymerize actin filaments,[21b] and this calcium dependent pathway is independent of the PKC pathway.[32] However, decreased gene expression of cofilin in iron-deficient cells is expected to result in declined actin depolymerization, whereas activation of PKC and increased intracellular calcium levels enhance actin depolymerization. The balance between these opposite effects possibly induces a relatively more stable fusion pore, so that a slightly higher fraction of release is observed with the 1st stimulation. In addition, phosphorylation of cofilin upon repetitive stimulation induces a less stable fusion pore with a slightly faster closure time, resulting in a smaller fraction of release.

Why does iron repletion fail to completely restore repetitive stimulation-induced decrease of fraction of release in iron deficient cells? One simple and likely explanation is that iron deficiency causes long-lasting alterations in neurotransmission.[33] A more interesting alternative is that the change of fraction of release upon repetitive stimulation observed here is due to the direct action of iron deficiency on plasticity. Previous studies of brain iron homeostasis have highlighted the role of iron deficiency in memory formation. Knocking out the cellular iron export protein, ferroportin1, induces iron deficiency in neurons in cortex and hippocampus, which negatively affects the formation of fear memory and the contextual fear response in mice.[34] Iron deficiency in foetal and early postnatal life also affects brain growth and development. For example, children with iron deficiency during early development have slower and smaller neurological and behavioural responses, despite subsequent treatment with adequate iron for 10 years.[35] It has been demonstrated that long-term potentiation and long-term depression are closely linked to memory storage, where activity-dependent long-lasting changes in synaptic strength give rise to the formation of memory in the brain.[36] Long-term potentiation is an activity-dependent form of synaptic plasticity, leading to a long-lasting enhancement of synaptic transmission. While long-term depression is an activity-dependent reduction that weakens the efficacy of synaptic transmission. Negative effects on plasticity would also influence the patterning of the neuronal network outputs in subsequently depression or facilitation.

Our study shows that repetitive stimulation is able to induce a change in the fraction of catecholamine released from vesicles in both control cells and cells with disruption of iron homeostasis, again supporting the hypothesis that the process of exocytosis involves partial release. This means that the number of molecules released during exocytosis can be fine-tuned, in contrast to the classic view of all-or-nothing release. The initiation and efficacy of synaptic plasticity might be interpreted as an alteration of the fraction of release at the cellular level, as the formation of memory is generally attributed to the modification of synaptic plasticity in neuronal networks. Iron deficiency and repetitive stimulation-induced changes in the fraction of release may disrupt one or more of the signalling cascades involved in plasticity and memory formation. A smaller fraction of release might not be sufficient for cells to respond on an input-modulated basis and is likely to cause impairment in memory formation. In addition, the ability of iron to modulate exocytotic neurotransmitter release might be involved in commonly observed behavioural modifications. For example, restless leg syndrome (RLS), a neurological-related movement disorder associated with abnormal sensations in legs, has been reported to be related to dopaminergic dysfunction and brain iron deficiency.[13a,37] This suggests that brain iron deficiency likely participates in the associated sensory-motor interaction and leads to the outcome of abnormal pattern of plasticity in RLS.[38] Thus, our study provides a possible link between the negative impact of iron deficiency on plasticity and iron deficiency-induced memory deficits. Moreover, it helps to improve the understanding of iron deficiency-associated diseases as well as plasticity and memory formation.

This study provides a fundamental link between iron deficiency, plasticity, and fractional release at the individual vesicle level, which helps to understand iron deficiency associated diseases as well as iron homeostasis and its regulation. However, a limitation associated with our methods is that single cell plasticity might be only a part of complex network-level interactions. From the perspective of electrochemistry, only electroactive species can be directly detected and quantified by SCA and IVIEC. In addition, amperometry detection lacks chemical selectivity, although selectivity is not necessary when comparing exocytosis to vesicle content to determine fraction released. In this respect, future work focusing on the coupling of high-resolution imaging techniques with electrochemical methods, including electrochemical biosensing techniques, would facilitate the discovery of the fundamental basis of neuronal communication and the understanding of the mechanism underlying plasticity, expanding their utilizations for neuroscience studies.

Conclusion

In conclusion, we investigated the effect of iron deficiency on plasticity using a repetitive stimulation paradigm. By combining electrochemical methods, SCA and IVIEC, changes in exocytotic transmitter release and vesicular transmitter content in response to repetitive stimulation were studied during disruption of iron homeostasis. Repetitive stimulation induces a less stable fusion pore during iron deficiency, leading to a decline in neurotransmitter release during exocytosis. However, the average amount of vesicular neurotransmitter storage remains unchanged after repetitive stimulation, resulting in a smaller fraction of release. With subsequent iron repletion, these changes are only partially reversible. It appears possible that the efficacy of plasticity correlates with the fraction of vesicular release. Many previous studies have demonstrated that iron deficiency causes learning and memory deficits caused. Based on our results, we suggest a mechanism where iron deficiency impacts plasticity in a negative manner by decreasing the

fraction of release upon repetitive stimulation, triggering dysregulation of neural networks. A smaller fraction of release may be insufficient for a regular signal transduction and thus influences the coordination of activities among neurons. This finding is particularly important as it provides a possible role of iron deficiency in modulating synaptic strength and yields new insights into the mechanism of how plasticity is impacted by iron deficiency at the cellular level. These data provide a framework for understanding the relation between iron deficiency and plasticity in model cells. If the exocytosis mechanism is conserved between these cells and neurons, this study would be crucial to explain cognitive disorders caused by brain iron disruption.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Exocytosis · Chemical Release Fraction · Iron Deficiency · Plasticity · Repetitive Stimulation

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