Master's Thesis

Learning in Single Cells

Computational Models of Habituation

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To my grandparents

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Abstract

Learning has typically been exclusively associated to neuronal organism. However, there is a great variety of data for learning in single tissue cells and single-celled organisms. In this thesis we focus on habituation, which is a non-associative form of learning and is commonly defined as a reversible decrease in response upon repetitive stimulation. On a cellular level, habituation has been observed in the ciliate *Stentor coeruleus* and in mammalian tissue cells. The significance of habituation lies in the ability to filter out non-harmful and irrelevant information, thereby allowing organisms to save resources for more important cognitive or cellular tasks. The dynamics of habituation and recovery is not solely determined by the number of previous stimuli but additionally depends on the frequency and intensity of stimulation. The aim of this thesis is to construct a biologically plausible model of habituation on a cellular level. The main focus is to explain how the frequency and intensity of stimulation determine the intracellular information processing and affect how fast systems habituate and recover. Following an idea of Staddon, we show that concatenation of two incoherent feed forward motifs (IFF) can explain the central effects of the stimulation frequency on the dynamics of habituation. The main feature thereby is the difference in timescales of the two concatenated motifs, which leads to a distinct pattern of memory buildup depending on the frequency of stimulation. Furthermore, our concatenated IFF model can account for the effects related to stimulation intensities and we propose a simplified model based on a single IFF motif, which shows the same qualitative behavior.

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1 Introduction

The ability to sense and respond to changes in the environment is one of the most crucial features of living organisms. In certain cases, the reaction to an environmental stimulus may not solely be determined by the stimulus itself, but additionally depend on previous experience of the organism. This - a persistent change in response to the same stimulus based on experience - might serve as a basic definition of learning.[1]

Two main types of learning are typically distinguished. Associative learning, with the most prominent example of Pavlovian conditioning, and non-associative learning such as habituation and sensitization. This work focuses on habituation, which is commonly defined as a reversible decrease in response upon repetitive stimulation. The significance of habituation lies in the ability to filter out non-harmful and irrelevant information, thereby allowing organisms to save resources for more important cognitive or cellular tasks.[2] Habituation has been extensively studied in neuronal organisms, which culminated in the definition of ten characteristic properties displayed by most, but not necessarily all, habituating organisms. (See table 1 for a complete list compiled by Rankin et.al.[2]) Even though these ten hallmarks of habituation have been crystallized from behavioral research, it is important to note that their definition is operational and can thus be applied to any organism with the ability to transiently respond to individual stimuli.

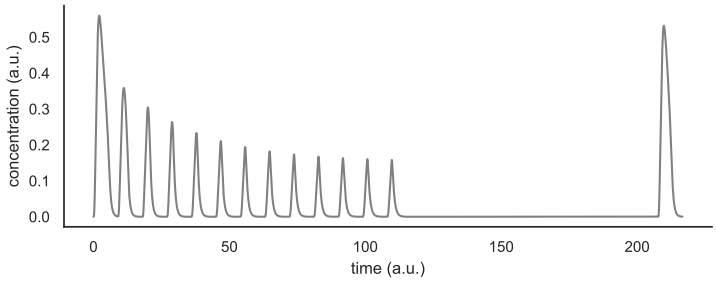

Figure 1: Example trajectory of a habituating and recovering system. 13 stimuli were applied until the system had reached its habituated level. Then the stimulus was withheld in order for the system to recover and a single test stimulus was applied after this recovery period.

Habituation is more than an artifact of sensory/motor fatigue or a cellular depletion mechanism. This is usually demonstrated by applying different types of stimuli and checking for dishabituation or stimulus specificity (see table 1).[3] One of the most startling hallmarks revealing the subtle information processing and time sensing involved in habituation is frequency sensitivity; the fact that habituation and recovery are faster for more frequent stimulation. This phenomenon suggests that habituating organisms evaluate the relevance of a stimulus not only based on the number of previous stimuli, but additionally extract information about its environmental context. The environment is less likely to change in between two tightly spaced stimuli and it is reasonable to assume that a non-harmful stim-

ulus can still be ignored after a short period of time. Following the same logic, the absence of stimuli after frequent stimulation results in a quick recovery.[4] Analogously, it is less fatal to ignore weaker stimuli, which explains why habituation is faster for less intense stimuli. This is another central hallmark referred to as intensity sensitivity.

Despite there are examples of associative[5,6] and habituation-like[7,8] learning in plants, learning has typically been exclusively attributed to networks of neurons in nervous systems, or more specifically to brains.[9] In these cases, learning has been shown to be accompanied by changes in gene expression in the involved neurons.[10] In the marine slug *Aplysia californica*, a species which due to its low number of large neurons has served as a model organism for the study of habituation, long-term habituation of the gill withdrawal reflex relies on the synthesis of new proteins in the presynaptic neuron, but also involves postsynaptic processes.[9] This is in stark contrast to short-term habituation, which has been traced back to habituation of neurotransmitter release in the sensory neuron and is therefore a purely presynaptic process.[11] This finding has been confirmed in species other than *Aplysia*[12,13] These molecular events suggest that the computations relevant for short-term habituation are performed within the sensory neuron - a single cell.

The most direct evidence for learning in single cells comes from experimental studies in unicellular organisms and mammalian tissue cells.[14] Early research on classical conditioning in the ciliates *Paramecium aurelia* and *Paramecium caudatum* dates back to the 1950s and 1970s.[15,16] Beatrice Gelber, whose work has recently been reviewed by Gershman et. al., trained *Paramecium* by repetitively presenting them with a syringe coated in bacteria. After several trials *Paramecium* swam towards the syringe, which served as the conditioned stimulus, even in the absence of bacteria. Gelber's experiments are also notable from a historical point of view since their controversial perception demonstrates the difficulties encountered by her and many others who followed the unorthodox path of studying learning outside the brain.[17,18] More recently, the list of organisms capable of associative learning has been extended to include *Amoeba proteus* and *Metamoeba leningradensis*.[19]

The evidence for non-associative learning in single cells is equally as rich. Herbert Spencer Jennings worked with *Stentor roeseli*, a trumpet shaped, sessile ciliate, which he irritated repeatedly. As a result he was able to observe a hierarchical sequence of avoidance behavior.[20] After a long period of skepticism and poorly performed attempts to disprove these findings, they have recently been successfully reproduced.[21] *Stentor's* avoidance behavior is remarkable because it shows that a single type of stimulus is not limited to quantitative modification of a given response, but can activate qualitatively different behavior in a hierarchical manner.

Most relevant for us, habituation has been observed in a variety of single-celled organisms such as the ciliate *Stentor coeruleus*[22,23] and the slime mold *Physarum polycephalum*.[24] Moreover, it has also been observed in non-neuronal mammalian tissue cells, including human embryonic kidney cells,[25] and in the rat adrenal pheochromocytoma cell line PC12,

where detailed studies were performed by Daniel Koshland. In these cells habituation and recovery of neurotransmitter release has been observed for different stimuli such as ATP, acetylcholine and high levels of potassium.[26–34] A list of the investigated hallmarks can be found in appendix A. Koshland's studies were carefully performed and rule out the possibility of a simple depletion mechanism by correcting for the loss of internal neurotransmitter during the experiment. Stimulus specificity between potassium and acetylcholine and dishabituation to phorbol esters further allow to exclude the possibility of fatigue.[26,27]

From the biochemical study of PC12 cells we know that habituation of neurotransmitter release is a downstream effect of the habituation of internal calcium levels.[28,32] The available data for stimulation with ATP suggests that this might be due to the downregulation of calcium influx.[32] However, different stimuli may rely on different pathways as can be inferred from the fact that habituation produced by potassium and acetylcholine stimulation are independent of each other.[27,30] The pathway activated by potassium stimulation has been hypothesized to involve L-type calcium channels and protein kinase C.[26] Despite these insights, the full circuit underlying habituation in PC12 cells has not yet been worked out in detail. Gaining theoretical understanding of the general mechanistic principles underlying habituation could further aid the search for biochemical mechanisms.

From a conceptual perspective, habituation can be implemented by a network including a memory species, which builds up either proportionally to the input or the response node and has an inhibitory effect on the latter. This corresponds to an incoherent feed forward (IFF) or a negative feedback (NF) loop, respectively (see figure 2). The memory species stores information about previous stimulation and accounts for the decrease in response through it's inhibitory effect on the response node. While it is relatively easy to construct a model for habituation and recovery, it is not trivial to account for the hallmarks of frequency and intensity sensitivity.

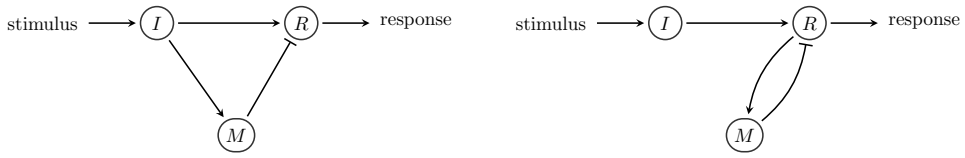

Figure 2: The incoherent feed forward IFF (left) and negative feedback NF (right) loop in the context of habituation. Both network motifs consist of a stimulus sensing input node (I) and a memory species (M) with inhibitory effect on the response node (R).

The IFF and NF network motifs are ubiquitous in nature[35] and therefore provide plausible building blocks for models on the cellular level. They have been shown to underlie adaptation; a phenomenon in which a persistent stimulus results in an initial increase in response followed by a subsequent decay back to the original steady state level.[36,37] Since adaptation and habituation are conceptually related (see figure **??**), adapting network topologies are a valuable source for the construction of habituating models. Detailed models of adaptation have been proposed in enzymatic and gene regulatory contexts.[38,39] One of the earliest

studied adapting systems is the chemotaxis of *Escherichia coli*, which has been shown to rely on the NF motif with the inhibitory memory being implemented in the form of multisite post-translational modifications.[18,40]

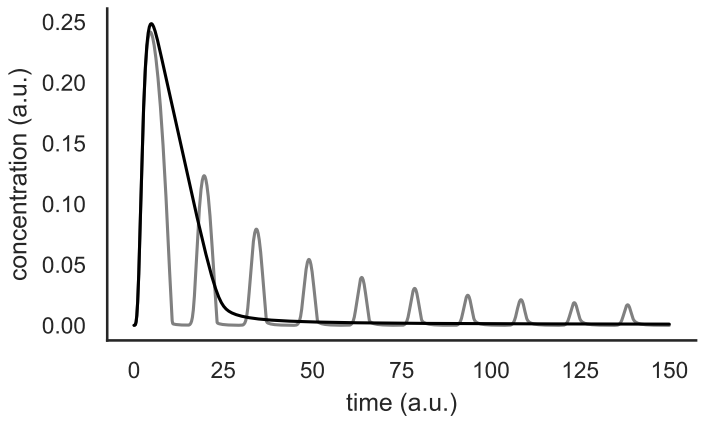

Figure 3: Illustration of the two related phenomena of adaptation (black) and habituation (gray). The two curves were obtained by numerical integration of the same ODE model with fixed parameters. For habituation the stimulus was applied with a period of 15 time units while adaptation was simulated with a persistent stimulus of the same intensity.

Two models of habituation have been proposed previously, both relying on the IFF motif. With the aim of providing a mechanistic framework for habituation without making any assumptions on the underlying molecular or neuronal substrates, in 2019 Bonzanni and others formulated a generalized model, which accounts for the basic properties of stimulus habituation and recovery, but fails to produce the less trivial hallmarks of frequency and intensity sensitivity.[41] This is in line with Staddon's argumentation that faster habituation and recovery for more frequent stimuli can be obtained with IFF motifs, but only if they are concatenated so that the output of the first motif serves as the input of the second.[4] By varying the properties of the memory species of the two modules, the first one being more rapidly decaying and "forgetful", less frequent stimuli can pass the first module without any substantial memory built-up. Habituation and recovery then mainly depend on the second module with longer lasting memory which recovers more slowly. Due to the limited scope of tested frequencies, the data presented by Staddon has to be treated with some care. (For a more detailed discussion see section 3.1.) However, in a second publication Staddon extended the idea of concatenation to NF motifs and more than two modules, which yielded more reliable results.[42] Since the idea of concatenation was implemented in a recursive, discrete time setting the question is to what extent this framework can be adopted in a cellular context using biologically plausible functions based on mass action and Michaelis Menten kinetics. To the best of our knowledge, no model exists addressing intensity sensitivity.

The aim of this thesis is to provide a biologically plausible model of habituation which accounts for the nontrivial hallmarks of frequency and intensity sensitivity. Following the idea of Staddon,[4] we show that concatenation of the IFF motif can generate the desired behavior in a molecular setting at the level of a single cell. The ubiquity of the IFF motif and the resulting generality of our model might facilitate the search for molecular substrates involved in habituation in various organisms. Studying the network topologies capable of habituation and the dependency on the frequency and intensity of stimulation has valuable applications in synthetic biology and biomedicine in situations of repeated stimulation such as drug treatments. Elucidating the mechanisms of learning from the perspective of single cells does not only enhance our understanding of the remarkable signal processing capabilities of cells, but also sheds light on the evolutionary origins of learning.

Table 1: The ten hallmarks of habituation from Rankin et.al.[2] Asterisks denote that the naming has been changed by us. The hallmarks investigated in this work are highlighted in grey.

Number	Name	Description
1	Habituation	Repeated application of a stimulus results in a progressive decrease in some parameter of a response to an asymptotic level.
2	Spontaneous recovery	If the stimulus is withheld after response decrement, the response recovers at least partially over the observation time.
3	Potentiation of habituation	After multiple series of stimulus repetitions and spontaneous recoveries, the response decrement becomes successively more rapid and/or more pronounced.
4	Frequency sensitivity*	Other things being equal, more frequent stimulation results in more rapid and/or more pronounced response decrement, and more rapid spontaneous recovery (if the decrement has reached asymptotic levels).
5	Intensity sensitivity	Within a stimulus modality, the less intense the stimulus, the more rapid and/or more pronounced the behavioral response decrement. Very intense stimuli may yield no significant observable response decrement.
6	Subliminal accumulation*	The effects of repeated stimulation may continue to accumulate even after the response has reached an asymptotic level [...]. This effect of stimulation beyond asymptotic levels can alter subsequent behavior, for example, by delaying the onset of spontaneous recovery.
7	Stimulus specificity*	Within the same stimulus modality, the response decrement shows some stimulus specificity.
8	Dishabituation	Presentation of a different stimulus results in an increase of the decremented response to the original stimulus.
9	Habituation of dishabituation	Upon repeated application of the dishabituating stimulus, the amount of dishabituation produced decreases.
10	Long-term habituation	Some stimulus repetition protocols may result in properties of the response decrement [...] that last hours, days or weeks.

2 Methods

The network topologies investigated in this work were implemented as ordinary differential equations (ODEs) which were built from biologically plausible mass action or Michaelis Menten functions. The ODEs were numerically integrated using the `odeint` function from the `SciPy` package in `python 3.8.12`.[43] Repetitive stimulation was simulated by a square wave input function with fixed intensity for stimulating periods and zero input for non-stimulating periods. The resulting integration trajectories were filtered for habituating behavior according to the filters listed in appendix B. Integration time was allocated on the fly and integration was stopped once the peaks did not change significantly anymore, which allowed for an economic allocation of computational resources.

In order to analyze a given network topology, a parameter scan with randomly generated parameter sets was performed. Since our main interest was in the hallmarks of frequency and intensity sensitivity, each parameter set was simulated with three different frequencies and intensities (9 combinations in total) for which habituation and recovery times were calculated. A network and it's parameter set were considered frequency sensitive if habituation and recovery times were strictly monotonically increasing with decreasing frequencies for at least one of the tested intensities. Analogously, intensity sensitive systems are strictly monotonically increasing in habituation time with increasing intensities for at least one of the tested frequencies.

For the analysis of frequency and intensity sensitivity, a reliable definition of habituation and recovery time is needed. Recovery times were calculated by integrating the systems without stimulation after the system has habituated. Test stimuli were independently applied at different times and the resulting peaks were compared to the initial peak. Once the test stimulus reached the initial peak level, the system was considered to have recovered and the time between the habituated stimulus and the test stimulus is referred to as recovery time. A detailed discussion of the definition of habituation time and the possible pitfalls is presented in the next section.

The main code for the generation of the random parameters and integration of the ODEs was developed by Ziyuan Zhao. Adjustments were made primarily for the algorithm to calculate habituation time, the filters and the formatting of the output files.

2.1 Definition of habituation time

In order to develop an automated computational framework for the study of frequency and intensity sensitivity it is necessary to quantitatively compare different habituation curves. Therefore, a definition of habituation and recovery time is needed. Intuitively, we say that a system has habituated if the response does not decrease significantly upon further stimulation. This can be formalized by calculating the relative difference, d, of neighboring peaks as displayed in equation (1).

$$d_i = \frac{p_i - p_{i+1}}{p_i} \tag{1}$$

Here, p_j denotes the jth peak and d_j is the relative difference between peak j and $j+1$. Habituation time is then defined as the number of applied stimuli until the relative differences fall below a fixed threshold. By default this threshold was set to 0.01. It is worth mentioning that habituation time is measured in number of stimuli and not in units of time.

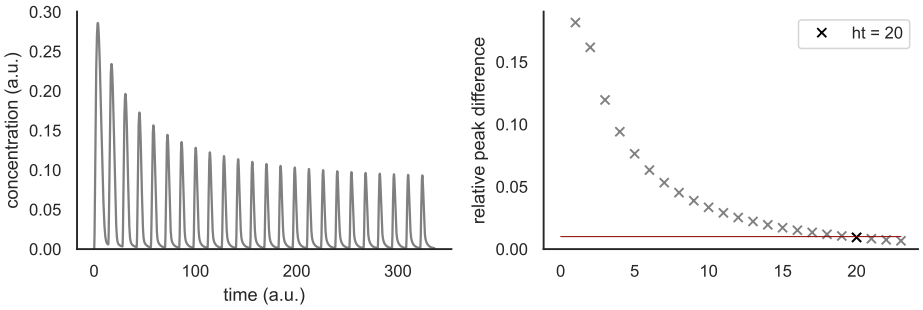

Figure 4: Definition of habituation time. Left: Response trajectory of a habituating system. Right: Relative differences between peaks. The red line marks the threshold of 0.01 and the black cross indicates the first peak difference below this threshold.

The definition applied in this work may not be equivalent to other explicit or implicit definitions found in experimental literature. In the standard reference of habituation Rankin et. al. state that more frequent or less intense stimulation result "in more rapid and/or more pronounced response decrement".[2] Even though they do not further specify how to quantify how "rapid" a response decreases, we assume that they refer to the decay rate associated with an exponential fit to the data. An explicit example of this definition can be found in Cheever and Koshland's publications about habituation of neurotransmitter release in PC12 cells.[31,32] In general, this definition can be problematic for two reasons. First, not all habituation data can be well approximated by an exponential function. In fact, Rankin et. al. state that "in many cases, the decrement is exponential, but it may also be linear".[2] Second, the exponential decay rate is strongly determined by the dynamics of the first few peaks and by how much the response decays in total while changes in later peaks are less significant. Therefore, exponential decay rates are not informative of the habituating behavior, which is determined by the dynamics of the later peaks.

In experimental literature, peaks are often normalized by the highest peak instead of displaying absolute values.[29] This can give rise to another difficulty when it comes to quantification of habituation time. Lets consider a system which for different stimulation frequencies shows very similar habituating behavior and the same habituation time but has different maximal peak levels. Such a system is depicted in figure 5 with a higher maximal peak level for more frequent stimulation. Even though both stimulation frequencies result in the same habituation time, the picture changes if we consider the normalized peak values. In this case, the peaks of the system with higher maximal peak level (upper panel in figure 5) have lower values when normalized compared to the system with lower maximal peak level. Therefore, also the absolute differences between neighboring normalized peaks are smaller. This can create the impression that the peaks are closer to each other and have already habituated. This is especially relevant for frequency sensitivity, since higher frequencies often show some degree of sensitization for the first few stimuli and therefore have higher maximal peak levels.[1] The fact that experimental data is often normalized can complicate a quantitative and reliable assessment of the hallmarks of frequency and intensity sensitivity.

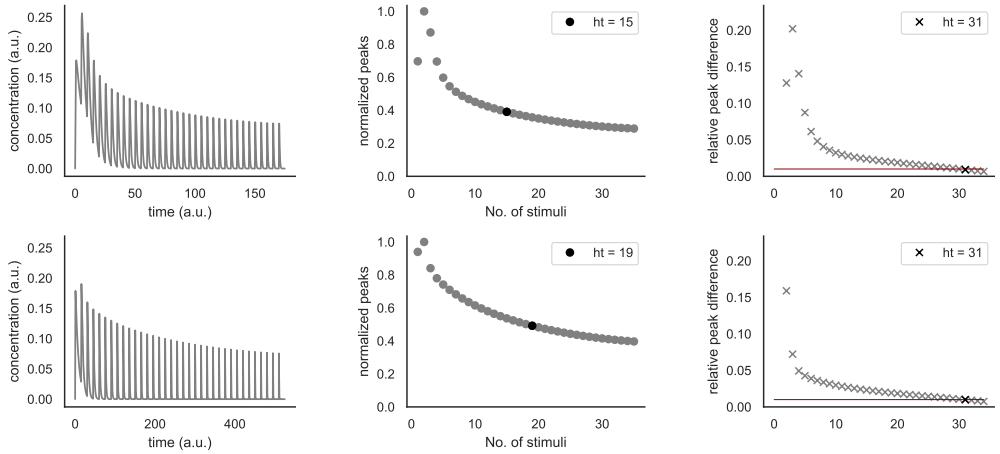

Figure 5: The upper panel shows data for a high stimulation frequency and the lower panel shows data for less frequent stimulation. Left: Habituation trajectory. Middle: Normalized peaks are depicted in gray and habituation time, calculated by max normalizing the peaks and applying a threshold of 0.01, is indicated with a black dot. Right: Habituation time was calculated with our standard definition of relative peak differences.

[1]The phenomenon that higher frequencies result in more sensitization was generally observed in our simulations and also reported and discussed from a behavioral perspective in Groves and Thompson 1970.[44]

Another difficulty arises when data is minmax normalized. Less frequent stimulation typically results in less pronounced (weaker) habituation.* Therefore, minmax normalization, which projects the maximal value to one and the minimal value to zero, artificially increases the distance between peaks. If habituation time is then calculated by applying a fixed threshold after minimax normalization, this results in higher habituation times for lower frequencies. Consequently, high habituation times are obtained for less frequent stimulation as a result of less pronounced habituation. Frequency sensitivity of minmax normalized peaks may therefore be solely an artifact of the level of final decrease relative to the highest peak. An example of this effect is given in figure 6.

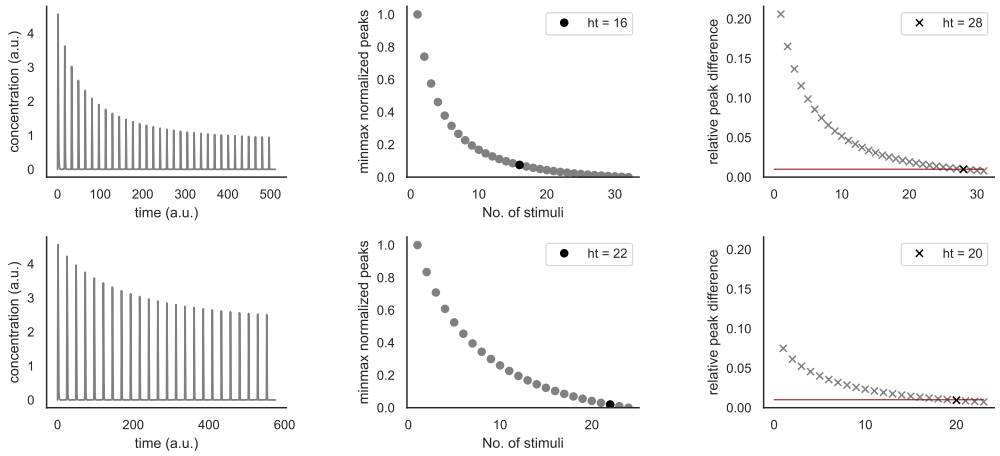

Figure 6: The upper panel shows data for a high stimulation frequency and the lower panel shows data for less frequent stimulation. Left: Habituation trajectory. Middle: Minmax normalized peaks are depicted in gray and habituation time, calculated by minmax normalizing the peaks and applying a threshold of 0.01, is indicated with a black dot. Right: Habituation time was calculated with our standard definition of relative peak differences.

In some extreme examples frequency sensitivity has been claimed even though only a few peaks are presented and they have clearly not yet reached their habituated level. [27] Assessing how "rapidly" a system habituates based on our definition of habituation time using relative peak differences is supposed to overcome the pitfalls presented here. It provides a quantitative measure which is independent of the shape of the curve and the strength of habituation. The definition used in this work is ideal for a computational treatment of habituation and its central hallmarks. Nevertheless, we are aware that it is difficult to apply our definition to experimental data due to fluctuations and measurement errors.

*This is a trend which we observed for both experimental and simulated data.

3 Results

The main goal of this project is to develop a theoretical model for habituation, explains how the central hallmarks of frequency and intensity sensitivity can arise from cellular networks. The evolution of these hallmarks is stunning, but not unsurprising, since they allow organisms to predict the relevance of a stimulus in the context of its environment. While the biological relevance of frequency and intensity sensitivity is evident, they are more difficult to explain from a conceptual perspective. Previously published attempts to model habituation and its central hallmarks are reviewed in the next section and are followed by a presentation of our own ODE models.

3.1 Previously Proposed Models of Habituation

The most recent model for habituation, which was published in 2019 by Bonzanni et. al., is based on a single IFF loop and provides a general framework for habituation and recovery without accounting for frequency or intensity sensitivity.[41] To the best of our knowledge, no model has been published which could explain intensity sensitivity. The situation is different for frequency sensitivity, which has been studied in a discrete-time setting by Staddon and Higga in the 1990s. Their original model was composed of two concatenated IFF motifs so that the output of the first motif serves as the input of the second motif. The main feature of the model is the difference in timescales between the two motifs. Following Staddon's argumentation, if the first memory decays more quickly, less frequent stimuli should pass the first motif without significant habituation. In this case, the behavior of the system would be dominated by the second motif with its slower decaying memory, which also takes more time to recover. The first module is therefore supposed to serve as a low-pass filter by decreasing its output for more frequent stimulation but letting lower frequencies pass unchanged.[4]

In the original paper from 1993, Staddon presents data for two different stimulation frequencies. From the replicated data presented in figure 7 it can be seen that more frequent stimulation indeed results in faster habituation and recovery. We then went on to generate data for a wider range of frequencies which were not presented in the original paper. This additional data tells a very different story. Except for the highest stimulation frequency (black curve in figure 7) the general trend of habituation and recovery times is reversed; more frequent stimulation result in slower habituation and slower recovery. Frequency sensitivity is therefore limited to the data presented by Staddon but does not extend to other frequencies.

In order to understand this apparent reversal of trends, it is important to note that the Staddon model operates with discrete time steps. The highest frequency chosen by Staddon was one stimulus per time step. In a discrete-time setting this implies that there is no time at which the stimulus is withheld. Even though we have to be careful with associating the behavior of a discrete-time model to actual biological processes, this might be interpreted as a persistent stimulus, which is not typically associated with habituation but with the

related phenomenon of adaptation. It therefore seems best to exclude the highest stimulation frequency and focus on frequencies which can more reliably be associated with habituation. In this case, frequency sensitivity is not observed for the given model and parameters.

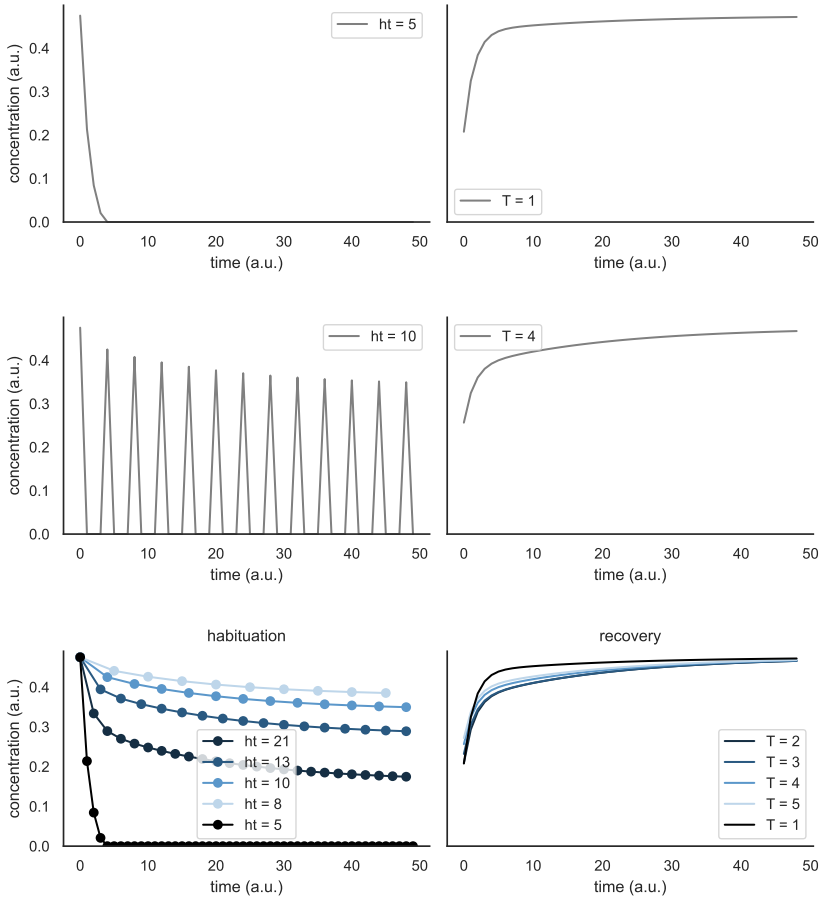

Figure 7: Reproduction of the concatenated IFF model in Staddon 1993.[4] The upper two figures show habituation (left) and recovery (right) trajectories for the two different stimulation frequencies, which were originally presented by Staddon. Habituation times are calculated using the definition of relative peak differences developed by us. The bottom figure shows habituation peaks and recovery trajectories for a wider range of stimulation frequencies.

In 1996 Staddon and Higga presented an updated version of their model based on the same idea of concatenation of IFF and NF motifs.[42] This time however, they concatenated up to ten motifs in a row and included NF loops. Additionally, they introduced a new quantity, reflex strength, which can take values below zero. This allows for more flexibility in the model and for habituation beyond zero, but comes at the cost that it can not be interpreted as a biological quantity. Therefore, the results obtained may not necessarily translate to a cellular context.

Figure 8 shows replicated data for three concatenated IFF motifs. The two frequencies tested here, which correspond to a true habituation stimulation protocol, show faster recovery for more frequent stimulation. Despite the fact that our data only qualitatively replicates the data presented in Staddon's paper, the trend of frequency sensitivity could be reproduced.

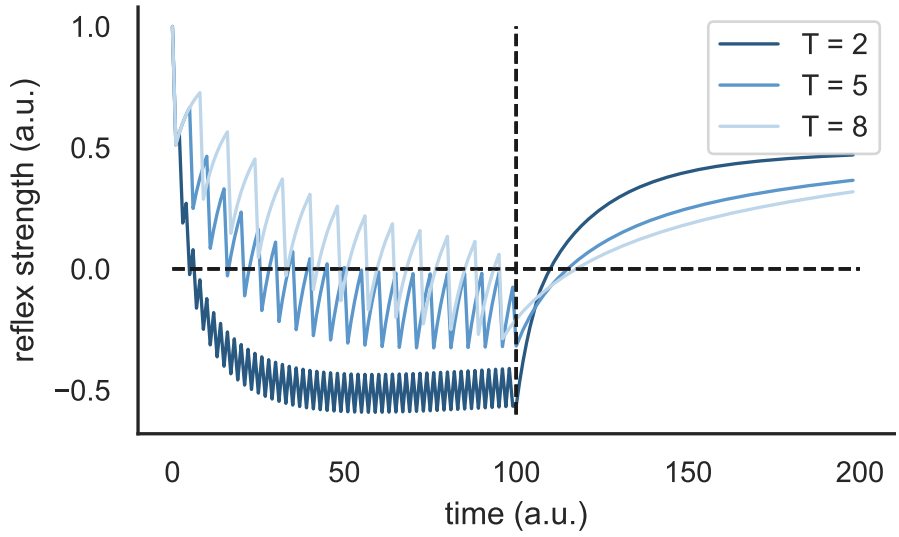

Figure 8: Reproduction of the data for three concatenated IFF motifs presented by Staddon and Higga in 1996.[42]

In conclusion, the work presented and reproduced here provides some evidence that concatenation of different habituating units can account for the nontrivial hallmark of frequency sensitivity. It remains to be shown whether this network topology can be translated to a biologically plausible, continuous-time setting. In the following sections we try to investigate this question. We further aim to expand the theoretical framework given here in order to provide a model for both frequency and intensity sensitivity.

3.2 An Enzymatic Model of Habituation from Concatenated Incoherent Feed Forward Motifs

In this section we present a deterministic ODE model for habituation. The model is generally based on enzymatic reactions which could for example be interpreted as post-translational modifications. However, no assumption is made on any specific molecular substrate or cell type. The model presented here therefore provides a general framework for the study of habituating systems and their potential underlying network architectures.

As depicted in figure 9 and equation (2) the model consists of two concatenated incoherent feed forward (IFF) loops and is built from mass action and Michaelis Menten kinetics. The exact implementation of the IFF motif was adapted from network architectures for adaptation proposed by Ma et. al. by taking advantage of the analogy between adaptation and habituation.[38] Each node represents a molecular species which can be either in its active (X_i) or inactive (X_i') state. The total concentration of active and inactive species $X_{tot} = X_i + X_i'$ is assumed to be constant. Without stimulation the system is at steady state if all molecular species are in their inactive state. Application of a stimulus, S, activates the input species (I) which in turn activates the memory (M) and the response (R). Additionally, the memory species enhances the degradation of the response species and therefore has an overall inhibiting effect on the response. The second motif is a copy of the first with the only difference that it is activated by the response of the first module rather than by the externally applied stimulus.

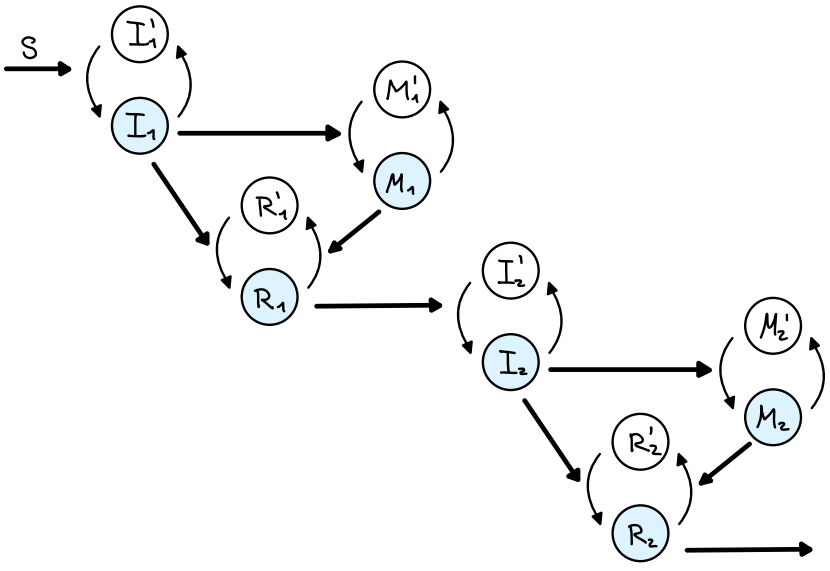

Figure 9: The concatenated incoherent feed forward (IFF) model. Active molecular species are highlighted in lightblue. The bold arrows indicate an activating effect on the reactions they point to.

$$
\begin{aligned}
\frac{dI_1}{dt} &= S * k_{Ia1}(I_{t,1} - I_1) - k_{Ii1} I_1 \\
\frac{dM_1}{dt} &= I_1 * k_{Ma1}(M_{t,1} - M_1) - k_{Mi1} \frac{M_1}{M_1 + K_{M1}} \\
\frac{dR_1}{dt} &= I_1 * k_{Ra1}(R_{t,1} - R_1) - M_1 * k_{Ri1} \frac{R_1}{R_1 + K_{R1}} \\
\frac{dI_2}{dt} &= R_1 * k_{Ia2}(I_{t,2} - I_2) - k_{Ii1} I_2 \\
\frac{dM_2}{dt} &= I_2 * k_{Ma2}(M_{t,2} - M_2) - k_{Mi1} \frac{M_2}{M_2 + K_{M2}} \\
\frac{dR_2}{dt} &= I_2 * k_{Ra2}(R_{t,2} - R_2) - M_2 * k_{Ri2} \frac{R_2}{R_2 + K_{R2}}
\end{aligned}
\tag{2}
$$

The system of ODEs was numerically integrated by applying a stimulus, S, which was kept at a constant intensity for the duration of stimulation, and was set to zero otherwise. Trajectories which were not classified as habituating were filtered out and the remaining systems were tested for frequency and intensity sensitivity. Figure 10 shows an example of a typical habituating response from the concatenated IFF model including the trajectories of all active species.

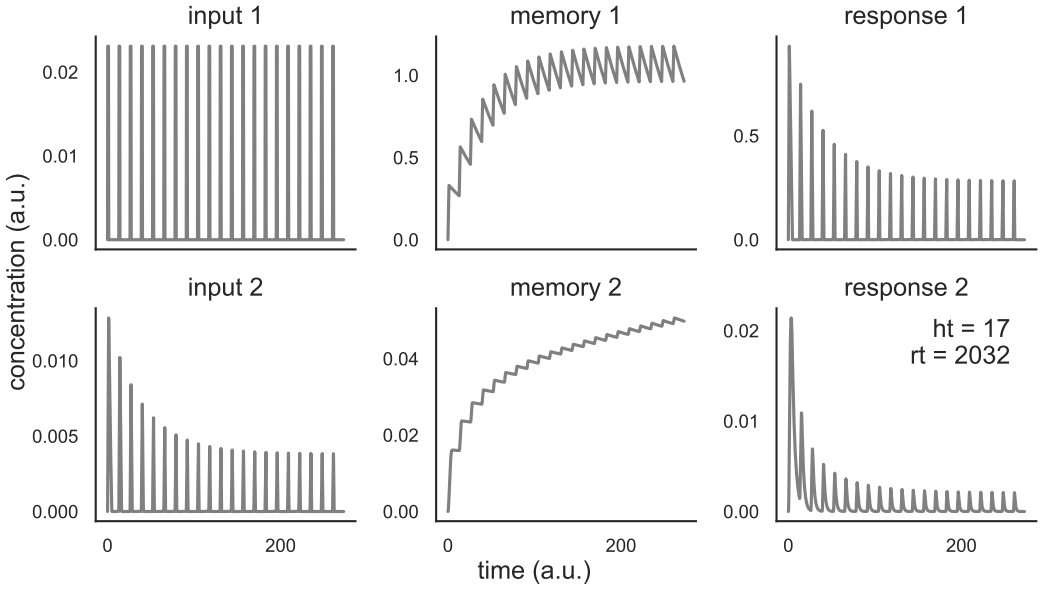

Figure 10: Trajectories of all active species of the concatenated IFF model. Habituation and recovery times are given on the bottom right. The system was simulated with a stimulation period of T = 13 and intensity I = 10 and the parameter values are listed in appendix C.

As expected, the concatenated IFF model shows habituation and recovery for suitable parameter sets. While the input nodes merely mimic the signal they receive and decay quickly, the memory species build up over time and store information about previous stimulation. Even though only the response node of the second motif was required to pass our habituation filters, also the first response shows a decrement typical for habituation.

3.2.1 The Concatenated Incoherent Feed Forward Model Shows Frequency and Intensity Sensitivity

Frequency and intensity sensitivity are the central hallmarks of habituation and have been reported in unicellular organisms and mammalian tissue cells.[22,25–27,32] In order to test whether our model can reproduce these hallmarks, each habituating parameter set was simulated with three different stimulation frequencies and intensities (nine combinations in total). Figure 11 shows the output trajectories for a given parameter set and selected stimulation frequencies and intensities.

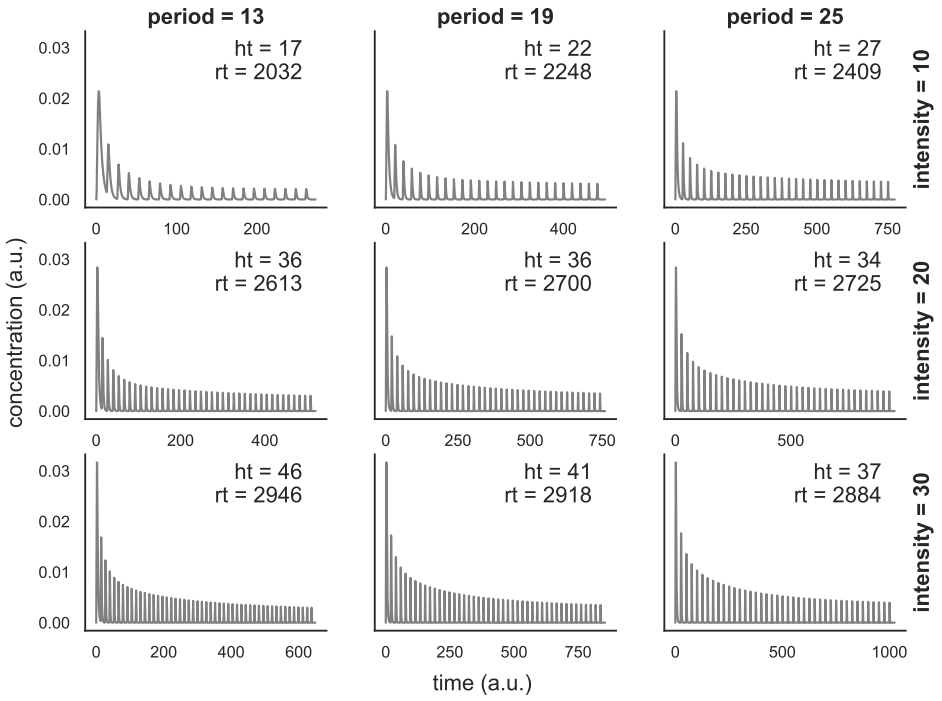

Figure 11: Response trajectories of the concatenated IFF model for different stimulation periods and intensities. Habituation and recovery times are indicated in the right upper corner of each plot. A full list of parameters can be found in appendix C.

For a stimulus intensity of I = 10, more frequent stimulation results in faster habituation and recovery. The concatenated IFF model therefore accounts for frequency sensitivity. Consequently, Staddon's discrete-time model can be adapted in a more realistic cellular context and account for frequency sensitivity. Furthermore, the concatenated IFF model additionally shows intensity sensitivity, which has not been studied by Staddon.

In addition to the effect of stimulus intensities on habituation time, we can also investigate the recovery dynamics. For the given parameter set, lower stimulus intensities also resulted in faster recovery. This trend has been observed for all the parameter sets which showed both frequency and intensity sensitivity. (Data not shown.) If the requirement for frequency sensitivity was lifted, only 101 out of 288 intensity sensitive parameter sets showed the same trend of faster habituation *and* recovery for lower stimuli. The dependence of recovery dynamics on stimulus intensities has not been investigated in single cells nor has it been reported in the standard reference of habituation.

3.2.2 Intermediate Stimulation Frequencies and Intensities

For reasons of computational feasibility it was necessary to restrict the number of tested frequencies and intensities in the parameter scans to three each. However, this does not guarantee that the trend of frequency and intensity sensitivity is maintained for intermediate values of T and I. To get a more complete picture the system presented in figure 11 was additionally simulated with intermediate frequencies and intensities.

As can be seen from table 2, habituation times are monotonically increasing for increasing stimulation periods within a range of $T = 13$ to $T = 25$. The trend of frequency sensitivity is therefore maintained for intermediate stimulation periods.

Table 2: Habituation times, ht, for different stimulation periods, T, for the concatenated IFF model. The stimulation intensity was set to $I = 10$ and all other parameters are listed in appendix C.

T	13	14	15	16	17	18	19	20	21	22	23	24	25
ht	17	17	18	18	19	21	22	24	25	25	26	26	27

Similarly, habituation time was calculated for intermediate stimulus intensities and the same trend of intensity sensitivity was observed. This holds for all tested stimulation periods as can be seen in table 3. While in principle the intensity of stimulation could take on arbitrary rational values, for convenience the analysis here is limited to even integers. Since monotonicity in habituation time was observed for all intensities and all tested stimulation periods, outliers may be unlikely. However, more randomized data would allow us to construct a more complete picture.

Table 3: Habituation times, ht, for different stimulation intensities, I, for the concatenated IFF model. The stimulation period was set to values of $T = 13, 19, 25$ and all other parameters are listed in appendix C.

I	10	12	14	16	18	20	22	24	26	28	30
ht (T = 13)	17	18	21	27	32	36	39	42	44	45	46
ht (T = 19)	22	26	30	32	34	36	38	39	40	40	41
ht (T = 21)	27	29	30	32	33	34	35	35	36	37	37

In conclusion, habituation times were monotonically increasing for increasing stimulus intensities and decreasing stimulation frequencies. Frequency and Intensity sensitivity, which were originally tested for only three selected values of T and I, are therefore preserved for intermediate stimulation parameters.

3.2.3 How Does the Threshold for Habituation Time Effect the Main Habituation Hallmarks?

By definition a system has habituated if the peaks do not decrease significantly upon further stimulation. In order to computationally assess this time point it was necessary to set a fixed threshold for relative peak differences. By default this threshold was set to 0.01. In order to test whether the hallmarks of frequency and intensity sensitivity are robust with respect to the habituation threshold, habituation times were recalculated with different threshold values.

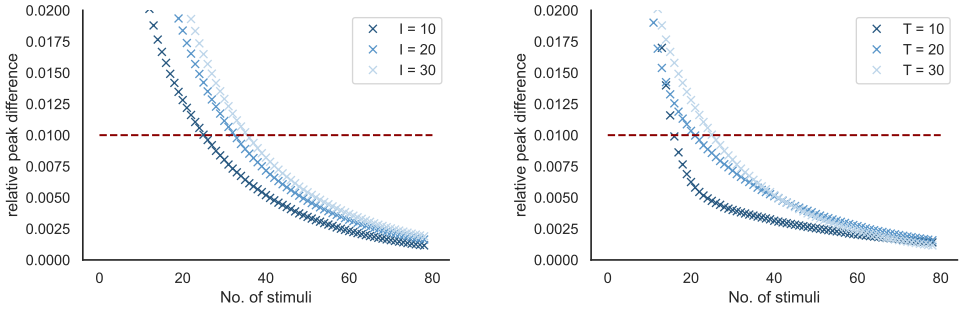

Figure 12: Relative peak differences with a fixed period of $T = 25$ (left) and fixed intensity $I = 30$ (right). The dotted red line indicates the original threshold of 0.01.

As can be seen from the data in table 4 intensity sensitivity is preserved for all tested thresholds. This is opposed to frequency sensitivity which was not observed for thresholds lower than 0.005. (See table 2.) From figure 12 it can be seen that the peaks decrease more quickly for the highest stimulation period of $T = 30$. This results in an intersection of peak differences for the different stimulation periods. Most likely this is due to the fact that memory levels did not reach a stable level within the simulated time span. Instead they keep increasing, which leads to continuing down-regulation of the response following uncontrollable dynamics. For future research it might therefore be advisable to include an

additional constraint to the parameter scans, requiring that the memory must reach a stable level.

Table 4: Habituation times calculated with different thresholds for a fixed stimulus period of $T = 25$. All parameters are listed in appendix C.

threshold	0.01	0.009	0.008	0.007	0.006	0.005	0.004	0.003	0.002
ht (I = 10)	27	29	32	35	38	42	48	55	65
ht (I = 20)	34	36	39	42	46	50	56	63	74
ht (I = 30)	37	39	42	45	49	54	59	67	78

Table 5: Habituation times calculated with different thresholds for a fixed stimulus intensity of $I = 13$. All parameters are listed in appendix C.

threshold	0.01	0.009	0.008	0.007	0.006	0.005	0.004	0.003	0.002
ht (T = 13)	17	18	19	20	22	25	32	44	62
ht (T = 19)	22	25	28	32	37	42	49	58	71
ht (T = 25)	27	29	32	35	38	42	48	54	65

3.2.4 Sensitivity Analysis

In nature, parameters such as catalytic rate constants and Michaelis-Menten constants are often subject to fluctuations due to changes in temperature, intracellular pH, or conformational changes and mutations of the corresponding enzymes. Maintaining unrestricted functionality under different conditions requires that the general behavior of cells must be unaffected by these parameter fluctuations. We performed a sensitivity analysis in order to test how much the selected parameters of the concatenated IFF model can be changed without annihilating frequency and intensity sensitivity. Each parameter was individually multiplied by a perturbation factor and the new system was tested for frequency and intensity sensitivity. The maximal perturbations which did not affect these hallmarks are displayed in figure 13 on a log_{10} scale.

The total concentration and the activation rate of the response species of motif 2 seem to be largely insensitive to changes. They can be modulated by as much as 10 or 0.1 times the original value and therefore span two orders of magnitude without affecting the general trend of frequency and intensity sensitivity. On the other hand, the parameters associated with the inactivation of memory species M_1 are most sensitive to perturbations.

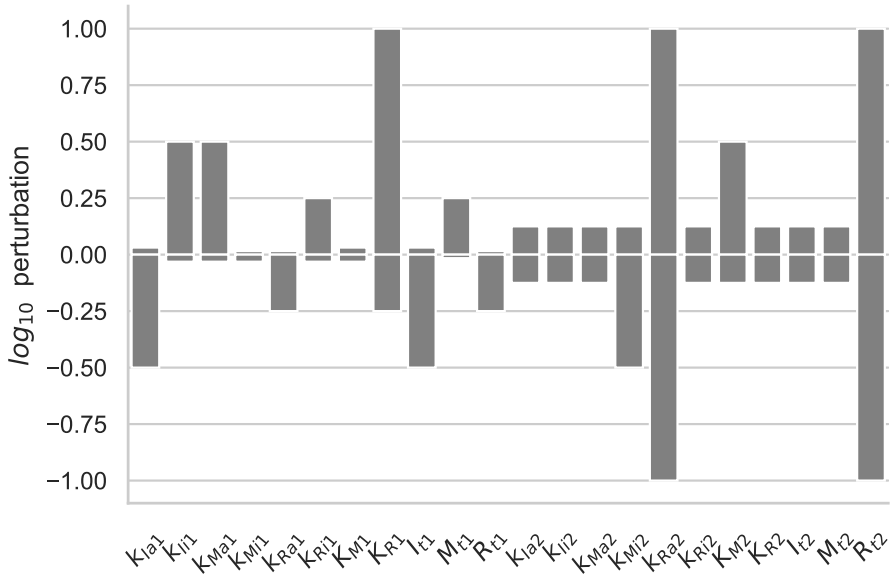

Figure 13: Sensitivity analysis. Maximal perturbations which still show frequency and intensity sensitivity are plotted on a log_{10} scale. Test periods are T = 13, 19, 25 and intensities are I = 10, 20, 30 and the original parameters are listed in appendix C.

3.2.5 The Recovery Dynamics Is Determined by Memory Levels

In his attempt to construct a rate sensitive model, Staddon proposed that the memories of the concatenated habituating motifs must operate on different time scales. If the memory of the first motif is "forgetful" and decays quickly enough, less frequent stimuli could pass the first motif without substantial response decrement. The stimulus would be passed on and recovery then mainly depended on the second module with longer lasting memory which recovers more slowly. Conversely, for high stimulation frequencies there is more memory buildup in the first module and less signal is passed on to the second motif. Therefore, Staddon suggested that in this case recovery would be mainly dependent on the second motif.[4]

In the concatenated IFF model presented here the rate of memory degradation of the first and second motif are 0.23 and 0.003, respectively. Therefore, the first decay rate of the first memory is indeed approximately one order of magnitude higher. The fact that the parameters associated with memory degradation are most sensitive to perturbations (as discussed in section 3.2.4) may further underpin the relevance of different memory life-spans.

However, the decay trajectories of both memory species reveal a slightly different picture. Due to the fact that the decay rate of the first memory is much faster, it is not surprising that the first memory reaches its initial level of zero more quickly. This clear difference in decay time between the two motifs is independent of the stimulation frequency. It seems therefore more likely that the recovery is always determined by the second memory, regardless of stimulation frequencies.

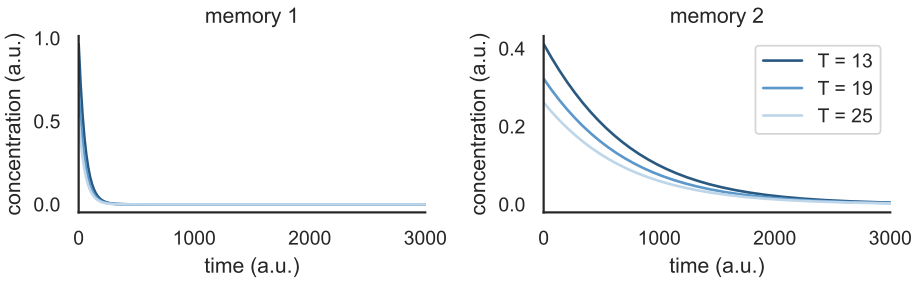

Figure 14: Decay of the two memory species from habituated levels for different stimulation frequencies. The stimulus intensity was set to $I = 10$ and the parameters are listed in appendix C.

Figure 15 shows the behavior of the memory species for different stimulation frequencies. For more frequent stimulation the first memory increases to higher levels than for less frequent stimulation. As a result, the response decrease is more pronounced for higher frequencies as displayed in the upper right panel. Consequently the second habituating motif receives less input which leads to a lower memory buildup. Since it is the memory of the second motif which determines the recovery, higher stimulation frequencies result in faster recovery. Frequency sensitivity is therefore the result of an inversion of memory levels.

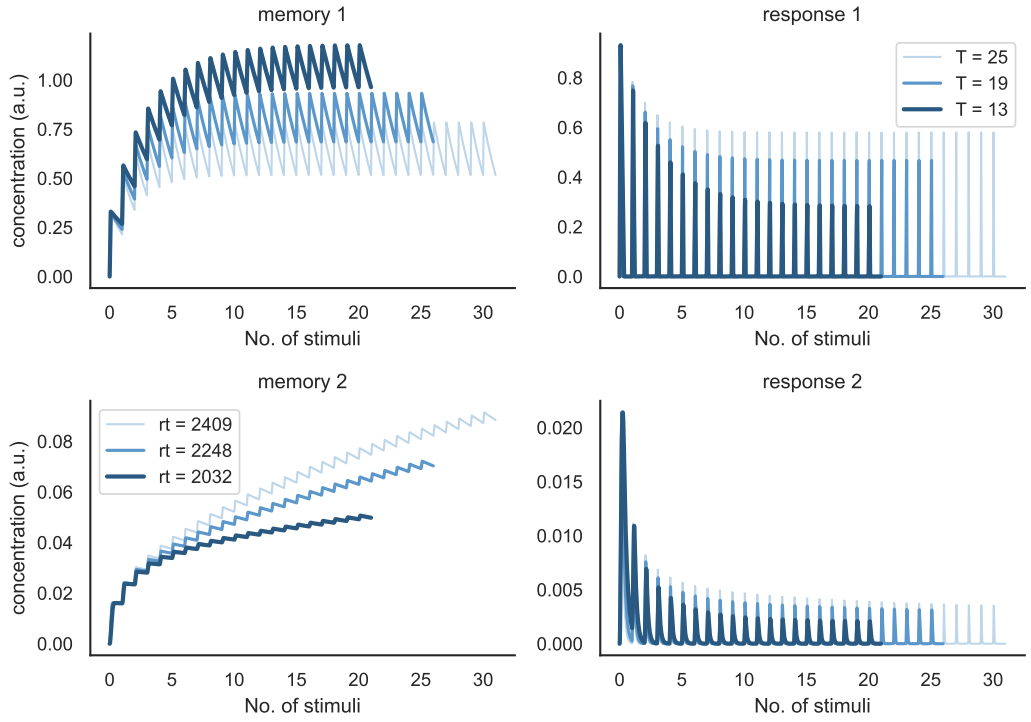

Figure 15: Trajectories of the concatenated IFF model for a fixed intensity of $I = 10$. The full parameter set is lited in appendix C.

3.3 Enzymatic Model of Habituation with Single Incoherent Feed Forward Motif

As showed in section 3.2.5 the key feature for frequency sensitivity is the reversal of memory levels as an effect of concatenation. This is necessary in order to get faster recovery for more frequent stimulation. To the best of our knowledge, the effect of stimulus intensity (rather than frequency) on the recovery dynamics has not been systematically reviewed. The hallmark of intensity sensitivity therefore only refers to the effect of faster habituation for less intense stimuli and recovery times are not considered. Due to this lower amount of constraints it may be assumed that network topologies that only account for may be less complex.

Figure 16 shows a model based on a single IFF loop. The structure of the IFF motif is the same as for the concatenated model with an input node (I) a memory species (M) and a response (R). As shown in figure 17 the single IFF model can account for intensity sensitivity. Within the scope of our parameter scans it was not possible to obtain frequency sensitivity with the single IFF model.

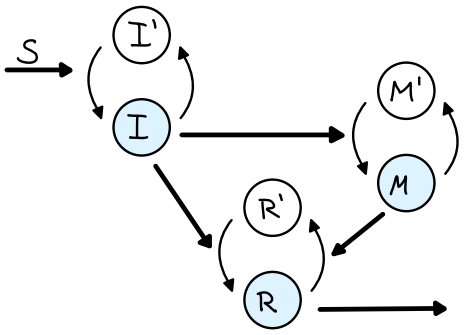

Figure 16: Incoherent feed forward (IFF) model. Active molecular species are highlighted in lightblue and the bold arrows indicate an activating effect on the reactions they point to.

$$\begin{aligned}
\frac{dI}{dt} &= S * k_{Ia}(I_t - I) - k_{Ii}I \\
\frac{dM}{dt} &= I * k_{Ma}(M_t - M) - k_{Mi}\frac{M}{M + K_M} \\
\frac{dR}{dt} &= I * k_{Ra}(R_t - R) - M * k_{Ri}\frac{R}{R + K_R}
\end{aligned} \tag{3}$$

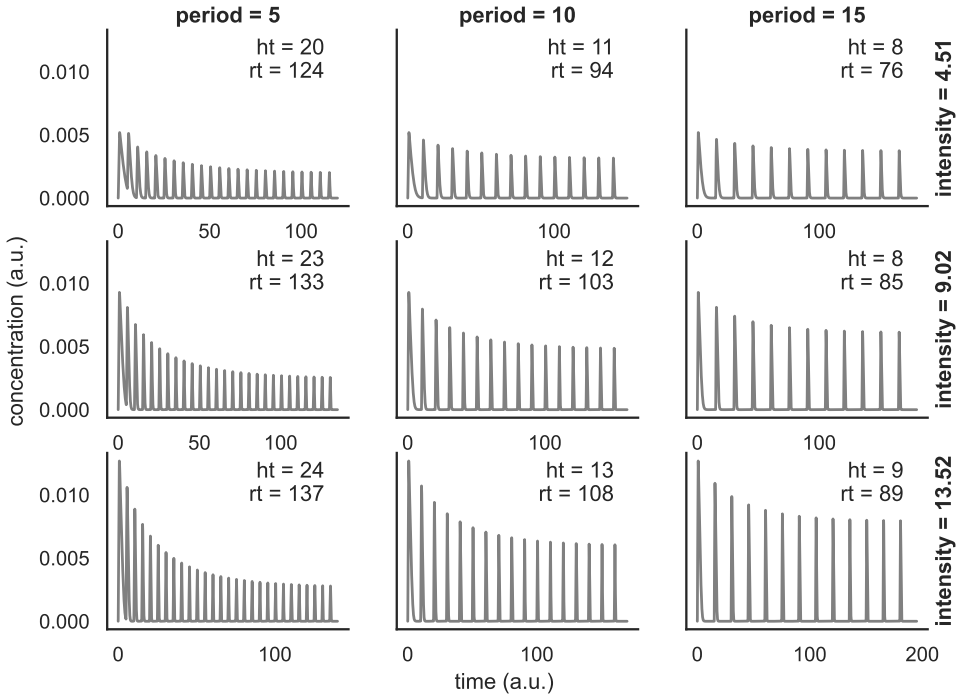

Figure 17: Response trajectories of the IFF model for different stimulation periods and intensities. Habituation and recovery times are indicated in the right upper corner of each plot. A list of all parameters can be found in appendix C.

4 Discussion

Despite the fact that learning is typically associated with neuronal organisms, there is a great amount of data for learning in single tissue cells and single-celled organisms.[17,21,22,30] This poses the question of how learning is implemented on a cellular level. In this study we provided a mechanistic model of habituation, a form of non-associative learning, which can be interpreted in a cellular context without relying on any specific molecular substrate. Conceptually, habituation and recovery are easy to obtain with an incoherent feed forward (IFF) or negative feedback (NF) network architecture. However, it is more difficult to account for the nontrivial hallmarks of frequency and intensity sensitivity.

In this work we showed that frequency sensitivity can be obtained by concatenation of two IFF motifs. The main feature of the model is a reversal of memory levels between the two motifs for different stimulation frequencies. Due to the fact that in our model the second motif is less forgetful and decays much slower, the recovery dynamics of the whole system is determined by the memory levels of the second motif. Within the scope of our study, this holds for all stimulation frequencies. This finding is opposed to Staddon's idea that for higher frequencies recovery depends on the memory dynamics of the first module. For suitable sets of parameters, more frequent stimulation results in a high level of memory species of the first, more peripheral IFF motif and consequently leads to more pronounced habituation. In turn, less input is passed on to the second motif, which therefore shows a low memory buildup and hence results in faster recovery. In addition to frequency sensitivity, the concatenated IFF model can also account for intensity sensitivity. Simplification of the model architecture to a single IFF motif allowed to maintain the hallmark of intensity sensitivity. However, an extensive search of the parameter space of the single IFF model did not result in frequency sensitive behavior.

The fact that the two different IFF motifs of the presented concatenated model were found to operate on different timescales, suggests that they might be implemented by different molecular substrates. Possible candidates could be receptor modifications, signalling pathways, post-translational modifications or gene regulatory networks.

In principle, reversal of memory levels can be achieved with a variety of different network architectures, as long as the separate modules show habituating behavior. Since single NF motifs can habituate (data not shown) they too are suitable building blocks for the construction of concatenated models. However, the concatenated NF models tested by us were computationally more difficult to integrate and it was not possible to obtain a parameter set that could account for frequency and intensity sensitivity. The models presented in this work are based on enzymatic transformations. IFF and NF motifs have been shown to underlie perfect adaptation also in a gene regulatory setting.[39] This suggests that in addition to our enzymatic model, a model of habituation could be implemented with IFF or NF motifs based on gene regulation.

Experimental data from PC12 cells suggest that receptor activation plays an important role in habituation.[26,31] It would therefore be worth investigating the habituating behavior of models based on different receptor states and how they could interact with more downstream processes. A first modeling attempt involving activation of a receptor was performed by Cheever and Koshland.[31] Their simple ODE model was based on toggling between an active and inactive receptor state but they did not investigate how the frequency and intensity of stimulation could affect the habituating behavior.

The parameter scans were mainly limited by the size of the parameter space, which made it unlikely to find a solution just by random sampling. In order to systematically investigate a broader variety of network architectures a biased random sampling algorithm could be used. This would require the definition of a scoring function, which quantifies how close or far the behavior of the system is from frequency and intensity sensitivity. A set of randomly selected starting parameters could then be modified and "pushed" towards a target score in order to obtain the intended behavior. This algorithm has successfully been applied to the study of transcription factor synergy and energy expenditure in gene regulation.[45,46]

While the models presented in this work operate on the level of a single cell, more general theories of habituation have been proposed in a neuronal context in behavioral literature. In general, two main ideas can be distinguished. The dual-process theory by Groves and Thompson emphasizes on the existence of two independent decremental and incremental processes, which account for habituation and sensitization, respectively.[44] The concatenated as well as the single IFF model can show initial sensitization prior to habituation for certain parameter sets (data not shown). This has primarily been observed for higher stimulation frequencies. A possible explanation may be found in the distinction between the positive and negative arm of the IFF loop. If the positive interaction between the input and response node is fast enough, the signal might be transmitted prior to any substantial memory buildup. If stimulation frequency is so high that there is not enough time for the response to fully decay between two stimuli, this could lead to sensitization. The main difference to the Groves-Thompson model is that there is no independent sensitization unit and sensitization is merely a property of the habituating IFF loop.

The second class of theories was developed by Sokolov, Konorski and Wagner and relies on the formation of an internal model, which is formed upon repetitive stimulation, and an arousal system, which senses and amplifies the stimulus.[47,48] The internal model exhibits an inhibitory interaction on the amplifying system. This framework has apparent parallels to an IFF motif architecture with the internal model acting as the inhibitory memory.

Internal models have recently been discussed in the context of learning from an information processing point of view.[18] The concept of internal models is useful in order to define learning in a more formal and potentially quantitative way. According to the definition of Gunawardena, learning is characterized by the formation of an internal model, which is required to change the behavior of the learning agent. The content of the internal model

may be quantified by the amount of mutual information between the learning system and its environment.[18]

Adaptation has been proposed to rely on the formation of an internal model.[18,49] Similarly, the memory buildup during habituation in our IFF-based model may be related to an internal model. In the context of habituation the internal model leads to a response decrement and therefore induces a change in behavior. While the internal model in systems which are based on NF motifs are reflexive, the IFF motif in our model could be seen as an instance of a reflective internal model.

The model presented in this work provides theoretical evidence for the idea that habituation can be implemented on the level of single cells and does not require networks of neurons. This does not only enhance our understanding of the remarkable signal processing capabilities of cells, but also sheds light on the evolutionary origins of learning.

A Hallmarks of habituation observed in PC12 cells

The following table lists all the hallmarks observed in PC12 cells. As discussed previously, the hallmarks 3-5 rely on the definition of habituation time. The list presented here is based on the (implicit) definitions used in the respective publications. For a more unified picture on the behavior of PC12 cells, a reevaluation of the presented data may be necessary.

Table 6: Reported hallmarks of habituation in PC12 cells. The measured response variable is norepinephrine release. Tested stimuli are ACh (acetylcholine), ATP (adenosine triphosphate) and K^+ (potassium).

#	**Hallmarks**	**Stimulus**	**Comments**
1	Habituation	K^+ [26,30]	
		ACh [27,30,31]	
		ATP [31,32]	
2	Spontaneous recovery	K^+ [26]	Only partial recovery.
		ACh [27,30,31]	Only partial recovery.
		ATP [31]	
3	Potentiation of habituation	K^+ [26]	Only partial recovery between trials.
		ACh [27,30,31]	Only partial recovery between trials.
		ATP [31]	
4	Frequency sensitivity	K^+ [26]	Stronger, but presumably not faster habituation for higher frequencies. Recovery was not tested. Data has been normalized.
		ACh [27]	Normalized data. Peaks have not habituated. Recovery was not tested.
		ATP [32]	Normalized data. Not all peaks have habituated. For higher frequencies habituation is more pronounced and more rapid. Recovery was not tested.
5	Intensity sensitivity	ATP [32]	Normalized data (Fig. 1). Indirect evidence by analogy between habituation and adaptation (Fig. 3).
6	Subliminal accumulation		Not reported.
7	Stimulus specificity	K^+/ACh [27,30]	K^+ and ACh habituate independently of each other (specificity).
		ATP/K^+ [32]	Stimulus generalization between ATP and K^+.
8	Dishabituation	K+ [26]	The drug Bay K 8644 and phorbol esters have been used as dishabituating stimuli.
		ACh [27]	Phorbol esters do NOT result in dishabituation to ACh stimuli.
9	Habituation of dishabituation		Not reported.
10	Long-term habituation		Not reported.

B List of filters for habituation curves

Since integration of our ODE models results in a wide variety of different output trajectories, we had to apply rules to filter for curves which show typical habituating behavior. For each period between two stimuli, the peaks and troughs were extracted as the max and min of the integration trajectory. This gives an array of peaks and troughs, respectively, on which the following filters have been applied.

1) The array of peaks must not be empty.
2) The highest peak must not be found later than at the third position. This allows for some sensitization for the very first stimuli.
3) The first peak must not be much lower than the highest peak. (By default not lower than 50% of highest peak.)
4) All peaks after the highest peak must be monotonically decreasing.
5) There must be at least two peaks after the highest peak.
6) There must be a substantial difference between the highest and the lowest peak. (By default at least 20% signal decrease.)
7) There must be a substantial difference between the first few peaks and troughs. (By default, the difference between each normalized peak and trough must be at least 0.05.) This serves the purpose of filtering out smoothly decaying curves.
8) Trough levels must not be too high. (By default not higher than 60% of the highest peak.)
9) The last trough must be almost zero. (By default not higher than 2% of the highest peak.)
10) The number of high troughs is limited. (By default there must not be more than five troughs which are higher than 10% of the highest peak.)

C Data of all figures

In this section all relevant parameters from the data in the main text are displayed. This allows to fully reconstruct the presented data. Initial conditions were 0 for all molecular species. The first four parameters refer to the period, the stimulation time, the minimal amplitude, which was usually set to 0, and the stimulus intensity. All other parameters follow the order given in the definition of the respective ODE models.

C.1 Figure 3

model: concatenated IFF
parameters: 0.024366646737364918, 0.6162464867015266, 0.04447485832575715, 0.01740099161185905, 96.9944876579579, 0.5336874306312571, 1.0372075152178233, 0.00753947591894144, 0.10731703147619494, 8.181386493165336, 0.013407828310457707, 18.58860238207617, 0.5855331556741393, 28.116952462518515, 0.0015082670835452668, 50.991885097545904, 0.2868800965666724, 0.002644047166350287, 0.0030896811525613125, 0.06495662117058498, 0.43515426472637364, 0.36355907347111044
periods: 15 and persistent stimulus
intensities: 9.44

C.2 Figure 5

model: IFF
parameters: 15, 0.1, 0, 2, 0.0011998378605234663, 16.04286162611607, 4248.959943311437, 0.00028620801653459794, 138.7363107916044, 2.0435827204559653, 0.413866889388133, 4.1405371446772685, 0.23655612645275295, 0.001308557895722929, 0.006360762971267835, 0.8306938814717175, 0.1346249191091918, 1.6171711451866124, 0.1068425066241821
periods: 5, 15
intensities: 2

C.3 Figure 6

model: concatenated IFF
parameters: 24, 0.8, 0, 19.86726732820633, 13.373357718016345, 42.06731234284933, 34.27749905900168, 0.709470020226804, 1.617258011909472, 10.327382175922727, 1.0758427047289987, 0.002091525419021543, 0.012467994713152632, 0.4118802578935219, 73.1381783415525, 0.02494742755106409, 20.21786858757745, 3.7388443875609076, 0.06098016936573249, 1.2777199403071084, 0.35032117082355596, 0.02982553631982044, 0.36133837105762495, 26.147111831335078, 23.76822568869142, 3.323672416939637
periods: 16, 24
intensities: 19.862726732820633

C.4 Figure 10, 11, 12, 13, 14 15

model: concatenated IFF
parameters: 13, 1.1111111111111112, 0, 10, 0.02320202797325348, 34.444923126046966, 4.216377156822992,0.23374762385737105, 21.343569877758522, 0.846886641845772, 12.652743115719046,0.0005245556966765035, 3.451779663144697, 3.2640433647323523, 2.5458652846171734, 0.004541338635022877, 8.733334023317827, 0.18909182921964465, 0.003144326356079373, 0.3565767612182425, 53.09936472431077, 2.000278819486687, 2.8497243825004994, 27.35741984065393, 2.732813518837399, 2.91549550812968
periods: 13, 19, 25
intensities: 10, 20, 30

C.5 Figure 17

model: IFF
parameters: 5, 0.5, 0, 4.508024302189586, 0.21431239109526712, 6.847671424297533, 0.004182136530799628, 0.8072215385326522, 0.004949310170350774, 98.04801954605847, 32.44935572372974, 0.002217674814821017, 2.380003203066648, 0.03652850995855296, 7.950356896778968, 0.19472486960861107, 1.4852248930584304, 0.015962998914022353, 0.003708017058489699, 3.314519158788617, 53.829283254737426, 0.10754566438231254, 7.088029916313852, 68.64017859545467, 84.99104335104246, 4.305905647453747

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