universität wien

MASTERARBEIT

Development of Behavioral Learning Paradigms in Mice Using Auditory Cues for Positive and Negative Reinforcement

Subpart: A Genetic Strategy to Identify Synaptically Coupled Neurons

angestrebter akademischer Grad

Master of Science (MSc)

Verfasserin / Verfasser:	Tanja Wernle
Matrikel-Nummer:	0016866
Studienrichtung (lt. Studienblatt):	A066834_Master Molekulare Biologie
Betreuerin / Betreuer:	Dr. Simon Rumpel

Wien, im 13.05.2010

Acknowledgements

First of all, I would like to thank my family, especially my mother, for continuous support during my studies as well as for encouraging me to purchase my dreams.

Moreover, I would like to thank Christina, Sylvia and Nicola for enriching conversations and continuous believe in me.

I would like to express my gratitude to Simon for being a great supervisor always encouraging me to pursue high aims and to look at things critically in order to make up my own mind about the ‘black box’ brain.

It was also a great pleasure to work together with Brice whom I would like to thank for fruitful discussions, programming as well as introducing me to the world of Matlab. Furthermore, I would like to thank Manuel for his support during cloning of all expression constructs, Juliane for introducing me in behavioral experiments and Bruno, Kaja, Jiss and Evi for being great colleagues and producing such a nice working atmosphere in the Rumpel lab.

Finally, I would like to thank people from the workshop, the mouse house facility, the IT department and the whole I.M.P. facilities for their support and producing such a great environment for high quality research.

Zusammenfassung

Unsere Hirnstrukturen sind aeusserst dynamisch und weisen ein hohes Mass an Plastizitaet auf, um Lernen und die Anpassung unseres Verhaltens an eine sich staendig veraendernde Umwelt zu ermoeglichen. In den letzten Jahren wurden vor allem im Bereich der Neurowissenschaften essentielle Beweise dafuer erbracht, dass das Grundprinzip jeglicher Erinnerung ‚synaptische Plastizitaet' ist, welches Aktivitaets bedingte Veraenderungen in der Staerke von neuronalen Verbindungen darstellt. Bislang ist jedoch nicht bekannt auf welche Weise diese neuronalen Veraenderungen auf Netzwerkebene organisiert werden. Es ist daher nowtwendig, um Lernprozesse und die Herausbildung von Gedaechtnisinhalten moeglichst genau untersuchen zu koennen, eine angemessene Anzahl an genau definierten Verhaltensexperimenten zu entwickeln,. Zusaetzlich sollte es moeglich sein neuronale Veraenderung auf Lernprozesse an sich oder auf nicht-assoziative Vorgaenge zurueckfuehren zu koennen.

Der erste Teil dieser Diplomarbeit beschreibt die Entwicklung von drei verschiedenenen Verhaltensexperimenten in welchen Maeuse spezifisch zu positiver bzw. negativer auditorischer Reizverstaerkung konditioniert wurden.

(1) Die Spezifitaet und Variabilitaet erlernter Reizantworten und damit verbundener Gedaechtnisinhalte ist entscheidend von der Art und Weise des zur Untersuchung verwendeten Verhaltens abhaengig. Aus diesem Grund fuehrte ich eine klassische auditorische Angstkonditionierung in Maeusen durch und untersuchte anschliessend zwei unterschiedliche Arten von Angstverahlten, Erstarren (‚freezing') und die Unterdrueckung fortwaehrenden Verhaltens, im speziellen des Trinkverhaltens (‚lick suppression'). Allgemein ist in Verhaltensexperimenten zu bereucksichtigen, dass Reizantworten sowohl durch den spezifisch konditionierten Reiz als auch durch nicht assoziative Aspekte der Konditionierung hervorgerufen werden koennen. Deshalb ist es notwendig die Spezifitaet jeglichen erlernten Verhaltens zu bestimmen. Zu diesem Zweck werden konditionierte Reizantworten mit Verhalten verglichen, das durch nicht konditionierte Reize ausgeloest wird. In meinen Experimenten konnte ich nach der Konditionionierung in allen Maeusen deutliches Angstverhalten feststellen. Allerdings war

die Spezifitaet der Reizantworten entscheidend von dem zur Untersuchung herangezogenen Verhalten abhaengig.

(2) Neuronale Plastizitaet wird entweder durch bestimmte Eigenschaften eines konditionierten Reizes oder durch die Art der verwendeten Reizverstaerkung hervorgerufen. Eine Moeglichkeit diese beiden Aspekte bestmoeglich unterscheiden zu koennen, ist die Konditionierung eines neutralen Reizes mit sowohl positiver als auch mit negativer Reizverstaerkung. Die Methode der operanten Konditionierung basiert ueblicherweise auf positiver Reizverstaerkung, und nimmt relativ viel Zeit in Anspruch, da neben einer einfachen Reiz-Belohnungs-Assoziation auch verschiedene Verhaltensregeln erlernt werden muessen. Im Gegensatz dazu ist die klassische Angstkonditionierung von vergleichsweise kurzer Dauer und eine robuste Reiz-Antwort kann bereits innerhalb weniger Minuten festgestellt werden. Dieser zeitliche Unterschied erschwert natuerlich den Vergleich von operanter und klassischer Konditionierung. Daher entwickelte ich, um die Dauer der operanten Konditionierung zu verkuerzen, ein Verhaltensexperiment, welches auf einer klassischen Mehrfach-Konditionierung zu einem ersten visuellen und einem zweiten auditorischen Reiz (‚second order conditioning') basierte. Die Trennung von prozeduralen und assoziativen Aspekten des Lernprozesses sollte eine beschleunigte Konditionierung zum zweiten auditorischen Reiz ermoeglichen. Ich konnte zeigen, dass alle untersuchten Mauese erfolgreich zu beiden Reizen konditioniert werden konnten. Allerdings, war nach einer einzigen Konditionierung zum auditorischen Reiz der Lernprozess noch nicht vollstaendig abgeschlossen.

(3) Verhaltensexperimente sollten einerseits robust und rasch durchfuehrbar sein, andererseits jedoch auch das Erlernen spezifischer Gedaechtnisinhalte ermoeglichen. Daher entwickelte ich ein disckriminatives operantes Verhaltensexperiment (‚go/no go task'), in welchem Maeuse spezifisch zu zwei komplexen Toenen konditioniert wurden, wobei jeweils nur ein Ton positiv verstaerkt wurde. Es konnten alle Mauese erfolgreich zu komplexen auditorischen Reizen konditioniert werden, jedoch war die Genauigkeit der Unterscheidung schwaecher als in aehnlichen, auf olfaktorischen Reizen basierenden Experimenten. Es ist daher anzunehmen, dass die Faehigkeit von Mauesen spezifische Reize zu unterscheiden betraechtlich von der jeweiligen sensorischen Modalitaet abhaengt.

Milliarden von vernetzten Neuronen, welche den unterschiedlichsten Zellarten angehoeren, bewerkstelligen im Gehirn eines jeden Saeugetieres Lernen und Gedaechtnis und erfuellen somit

die verschiedensten Funktionen. Um einen besseren Einblick in besagte Komplexitaet zu gewinnen und die zugrunde liegenden neuronalen Netzwerkmechanismen besser zu verstehen, waere es von Vorteil ein einziges Neuron mitsamt seiner praesynatpischen Verbindungen untersuchen zu koennen. Bislang ist es jedoch, neben den unterschiedlichsten Methoden zur Visualisierung von neuronalen Verbindungen, noch nicht moeglich selektiv ein einziges Neuron und spezifisch dessen praesynatpische Verbindungen zu untersuchen.

Der zweite Teil dieser Diplomarbeit beschreibt die Entwicklung von Expressions Vektoren, welche die Expression zahlreicher Varianten eines Fusionsproteins zwischen Cre-Recombinase und des schweren Strangs des Tetanus Toxins ermoeglichen. Der zugrunde liegende Gedanke ist, die Vielseitigkeit und Flexibilitaet der Cre-Rekombinase mit den spezifischen retrograden Transporteigenschaften des Tetanus Toxins zu verbinden und somit spezifisch alle praesynaptischen Verbindungen eines einzigen Neurons bestimmen zu koennen. In naher Zukunft sollte die Anwendung eines solchen Fusionproteins in Verbindung mit Elektroporation eines einzelnen Neurons es ermoeglichen neuronale Netzwerkverbindungen in transgenen Maeusen zu untersuchen, welche eine Cre-Rekombinase bedingte Expression eines Reportergens aufweisen.

Abstract

Our brains are highly dynamic and plastic in structure in order to allow learning and adaption of behavior to a constantly changing environment. Recent years of research found substantial evidence that the substrate of memory is ‘synaptic plasticity’ which represents activity dependent changes in strength of neuronal network connections However, little is known about the organization of such neural changes on circuit and network level. Therefore, it is essential to establish a specific set of well defined behavioral learning paradigms that allow the sensitive measurement of memory and its specificity. Furthermore, it is important to be able to dissociate between learning-related neuronal plasticity and simply non-associative processes.

The first part of this thesis describes the establishment of three behavioral learning paradigms in mice including positive and negative reinforcement of complex auditory stimuli.

(1) The specificity and variability of the memory assessment depends crucially on particular properties of the behavior used as read out. Therefore, I tested auditory fear conditioning to complex sounds in mice using freezing and lick suppression as two alternative behavioral memory read outs. I find that after conditioning mice show robust fear responses for both behaviors. However, the specificity of the memory, as measured by the induction of fear behavior by stimuli that were not used for conditioned, is dependent on the behavioral assay.

(2) A strategy to dissociate neuronal plasticity induced by the way a particular stimulus was reinforced from neuronal plasticity that is induced by particular conditioned stimuli itself is to compare learning paradigms involving either positive or negative reinforcement for the same stimulus. Typically, operant conditioning paradigms involving positive reinforcement are time consuming since they include learning of several procedural aspects of the task beyond the simple stimulus→reward association. This makes the comparison between operant and classical fear conditioning, which usually occurs within the order of minutes, difficult. Here, I established a learning paradigm based on classical second-order conditioning using visual and auditory stimuli as salient cues for reward. This allowed the temporal separation of procedural and associative aspects of learning thus accelerating operant conditioning. I find that in this learning

paradigm mice reliably form the association of sound to reward and that initial, but not complete, learning occurs within the first conditioning session.

(3) Beside the implementation of fast learning paradigms that allow sensitive measurement of memory it is also important to assess the specificity of the memory formation. I tested behavioral responses of mice specific to properties of conditioned sound stimuli through the establishment of an auditory operant go/no-go discrimination task in which one of two complex sounds was positively reinforced. I find that mice can be trained to discriminate between two complex sounds, however, the fraction of mice achieving high performance levels is low compared to similar olfactory learning paradigms. Therefore, it seems that discrimination of stimuli of different modalities might pose different challenging tasks for mice.

In general, learning and memory is mediated in the mammalian brain through billions of neurons which are highly variable in cell type and function and interconnected in complex networks. A major step towards a better understanding of neuronal circuit computations would be the ability to map out all the input neurons to a given postsynaptic neuron. Despite considerable progress in labeling interconnected neurons, a system sensitive enough to selectively label input neurons of a single target neuron is still lacking.

The second part of this thesis describes the design and construction of expression vectors that allow the expression of variants of a fusion protein of the tetanus toxin heavy chain and Cre-recombinase. The idea is to take advantage of the sensitivity and versatility of Cre-recombinase and the selective transsynaptic transport of tetanus toxin for specific identification of input neurons. In the future, these constructs will hopefully allow identification of synaptically coupled neurons after electroporation of single neurons in a transgenic mouse allowing conditional expression of a fluorescent protein depending on Cre mediated recombination.

Table of Contents

1. Development of behavioral paradigms in mice using auditory cues for positive and negative reinforcement

1.1 Introduction

Our brains are highly dynamic and plastic in structure in order to allow learning and adaption of behavior to a constantly changing environment. Therefore, billions of neurons are synaptically coupled in large networks which simultaneously integrate and retrieve information. Typically, one single cortical neuron receives up to 10.000 synaptic inputs and transfers information encoded in an action potential only, if approximately 100 of its synapses are activated at the same time. Thus, learning, memory and neural network computations are mediated by changes in synaptic efficacy (Schafe, Nader et al. 2001). Already in 1949 Donald Hebb proposed that 'when two interconnected neurons fire at the same time, the synapses between them become stronger, and remain stronger for a long time afterwards' (Maren 2005). Nowadays, the most consistent link between synaptic plasticity and memory formation has been formulated by Morris and colleagues in 2000 as the synaptic plasticity and memory (SPM) hypothesis (Neves, Cooke et al. 2008):

'Activity dependent synaptic plasticity is induced at appropriate synapses during memory formation, and is necessary and sufficient for the information storage underlying the type of memory mediated by the brain area in which that plasticity is observed.'

1.1.1 The expression of associative memories at cellular and molecular level

The leading cellular model for associative memory proposes is long-term potentiation of synaptic transmission (LTP) (Martin, Grimwood et al. 2000) . On the molecular level, LTP is induced by the insertion of amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors containing the GLUR1 subunit into the postsynaptic membrane which finally leads to an enhancement of current influx (Shi, Hayashi et al. 2001). In order to encode memory traces, LTP needs to exhibit two basic properties: First, it should be associative, which means that weak synaptic connections are strengthened through co-activation of weak and strong inputs on one

neuron at the same time. Second, considering that one neuron receives up to 10.000 input connections, LTP needs to occur specifically at synapses of active but not inactive, presynaptic afferents to the postsynaptic cell. Furthermore, the induction of LTP at a given synapse is critically dependent on two receptor types in the postsynaptic membrane: *N*-methyl *D*-aspartate-receptors (NMDAR's) and L-type voltage-gated-calcium-channels (VGGC's) (Blair, Schafe et al. 2001).

NMDAR's are ligand-gated ion channels which are typically blocked by a magnesium ion at resting membrane potential. However, if presynaptic stimulation leads to binding of glutamate and simultaneously postsynaptic depolarization occurs, the channel opens. Therefore, it is an excellent coincidence detector for synaptic activity at specific synapses of interconnected neurons. After opening, potassium and calcium ions enter the cell. Calcium is an important second messenger molecule mediating gene transcription through activation of several kinase-specific-pathways involving Ca-calmodulin-dependent-kinase (CAMKII), mitogen-activated-protein-kinase (MAPK) and Protein kinase A (PKA). However, calcium influx through NDMAR's is not sufficient to initiate specific signal cascades. Therefore, L-type VGCC's are also essential for LTP induction. These channels are characterized by a high activation threshold and open only upon strong depolarization of the postsynaptic cell. Typically, this occurs when the cell fires a spike and the action potential back-propagates (BPAPS) from the soma into dendrites of the post-synaptic cell. When BPAPs collide with excitatory post-synaptic currents (EPSPs) at active synapses they are prolonged and amplified. Finally, this allows synaptic specificity of LTP (Martin, Grimwood et al. 2000; Blair, Schafe et al. 2001).

In general, memories are stored in associative networks over different time scales. For example, short-term memory is based upon transient enhancement of synaptic transmission and mainly mediated by local recruitment of AMPA receptors. In order for long-term persistence of these changes, i.e. their consolidation, processes are initiated that critically dependent on macromolecular protein synthesis (Frey and Morris 1997).

1.1.2 Learning induced changes in auditory subcortical and cortical areas

Plasticity in the brain is not only a focus of research at the cellular level, but also on the level of neuronal circuits. In particular learning paradigms involving the auditory modality had been investigated successfully in the past. Typically, an auditory sensory neuron responds to a limited frequency band, its receptive field, and shows highest response amplitudes to a single so called best frequency. Furthermore, neurons in subcortical and cortical areas are responsive to a variety of sound properties such as frequency, duration and amplitude. The organization of receptive fields within the cochlea is also reflected across the surface of the primary auditory cortex (A1) giving rise to a tonotopic map of sound frequencies. In general, auditory stimuli reach the cortex in an already highly processed manner. Several subcortical relay stations such as the cochlear nucleus, the inferior colliculus, and the auditory thalamus process information (King and Nelken 2009).

Memory traces in subcortical and cortical areas are expressed by receptive field plasticity of neurons. They can shift their tuning towards behavioral important frequencies which leads to an increase in area of their representation and changes in tonotopic maps. Weinberger et al. showed that immediately after learning receptive field plasticity develops in the ventral and medial geniculate body of the auditory thalamus as well as in auditory cortical areas (Weinberger 2004).

1.1.3 Learning induced plasticity in the amygdala

The amygdala is an almond-like shaped structure in the medial temporal lobe and plays a crucial role during memory formation of fear-related contents. It was shown in several studies using auditory fear conditioning in mice that information about the auditory and the aversive shock stimulus converge on the lateral amygdala and induce there synaptic plasticity (Romanski and LeDoux 1992; Fanselow and LeDoux 1999; LeDoux 2000; Rumpel, LeDoux et al. 2005; LeDoux 2007). Furthermore, cortical areas which are both afferent and efferent to the amygdala participate in storage and encoding of specific aspects of fear memories. For example contextual cue information is mainly processed in the hippocampus (Fanselow and LeDoux 1999; Sara 2000; Bouton, Westbrook et al. 2006).

1.1.4 Behavioral learning paradigms in auditory conditioning

Activity dependent synaptic plasticity is fundamental for creating and storing memory traces. However, changes in neural activity can relate to associative learning processes or simply present non-associative processes. Therefore, it is essential to link synaptic plasticity to specific behavioral outputs in order to assess learning induced neural changes. In typical behavioral learning paradigms animals need to associate a specific stimulus with a subsequent event (classical conditioning) or their behavior with following consequences (operant conditioning).

1.1.5 Classical Conditioning

Classical conditioning is typically mentioned in context with Ivan Pavlov's famous experiments on dog physiology in the 1920ies. He paired an initial neutral auditory stimulus of a metronome repeatedly with food. Therefore, it acquired the ability to elicit salivation in the dog without any food delivery (Pavlov 1927). This learning paradigm was later expanded and is now well known as 'Pavlovian Fear Conditioning'. Typically, a neutral conditioned stimulus (CS) is paired with an unconditioned aversive stimulus (US) and acquires the capacity to elicit unconditioned fear responses (UR). Innate fear responses e.g. freezing, physiological alterations e.g. increase in heart rate or more complex fear responses e.g. suppression of ongoing behavior are used as behavioral memory read out. In Pavlovian fear conditioning, typically freezing is used as memory read out since it is a robust and rapidly encoded behavior. Furthermore, it relies on molecular mechanisms that support long-term synaptic plasticity in the brain and the amygdala represents an essential part of the fear circuitry (LeDoux 2000; Maren 2001; Maren and Quirk 2004). In most auditory fear conditioning studies pure tones are used as auditory stimuli. However, it is very unlikely that mice experience single pure tone frequencies in their natural environment. Therefore, little is known about encoding and processing of complex sounds in the auditory cortex.

1.1.6 Second order conditioning

Memories are rather stored in interrelated complex networks than in isolated manner. Therefore, second order conditioning learning paradigms are ideally suited to investigate underlying neural computations (Debiec, Doyere et al. 2006). Typically, in the first-order phase a neutral stimulus (CS1) is paired with an unconditioned stimulus (US) and in the second-order phase a second neutral stimulus (CS2) is paired with CS1. Auditory, visual or olfactory cues can be used as neutral stimuli and the US is commonly presented by a foot shock similar to Pavlovian fear conditioning. Consequently, after pairing CS1 and CS2 the animal exhibits to CS2 a conditioned response although it was never directly paired with US (Gewirtz and Davis 1997; Gewirtz and Davis 2000. The underlying associative network between CS2→CS1→US has been formed and the internal presentation of CS2 is based on the motivational state elicited by the US rather than the US itself (Paschall and Davis 2002).

1.1.7 Operant conditioning

In the outside world presumably little learning involves direct pairing of events with powerful, unconditioned reinforcers. It is more likely that consequences are associated with a specific behavior and subsequently animals operate direct on the environment in order to elicit specific consequences (Lorenzetti, Baxter et al. 2008). The term ‘operant behavior’ was already introduced in the 1930ies by B.F. Skinner who clearly separated this term from adaptive animal reflexes. Furthermore, learning about consequences does not necessarily implement any purpose of behavior. Already in 1911 E.L. Thorndike postulated in his ‘Law of Effect’ that ‘the approximate simultaneous occurrence of a response and certain environmental events, changes the responding of the organism, increasing the probability that responses of the same sort will occur again’. Skinner added the small detail that responses were simply not less likely to occur after they had been followed by a certain consequence (Skinner 1984). Finally, applying Darwin’s theory of natural selection on operant behavior proposes that organisms with advantageous behavior are more likely to produce descendants than others. Therefore, an intrinsic phylogenetic purpose replaces intentional animal behavior.

1.1.8 Discrimination conditioning

Understanding the adaptation of behavior to a constantly changing environment implies the investigation of basic sensory information processing. Typically, the underlying neural mechanisms depend on the time needed for stimulus integration as well as on stimulus similarity. Therefore, reaction times of responses to a given sensory stimulus are commonly used as behavioral read out. In order to test learning and discrimination in mice typically operant go/no-go learning paradigms are used. Two different stimuli of varying similarity are presented either with reward (S+) or not (S-) and subsequently animals need to discriminate between the two stimuli in order to receive reward. So far, most studies in mice have focused on the investigation of sensory processing in the olfactory bulb. Typically, animals need to discriminate between odors of different complexity in order to receive reward. The accuracy of performance and the time needed for decision making is measured as behavioral read out (Abraham, Spors et al. 2004; Rinberg, Koulakov et al. 2006). However, little is known about sensory processing and integration of complex sounds in the auditory cortex of mice. Furthermore, few studies have focused on discrimination of auditory stimuli and were mainly performed in rats using frequency modulated pure tones (Ono, Kudoh et al. 2006; Rybalko, Suta et al. 2006).

1.1.9 Central question

Despite the fact that a multitude of learning paradigms had been applied to various species in the past, a consistent set of paradigms with high sensitivity for memory formation in mice involving complex auditory stimuli, that resemble naturally occurring stimuli, is still lacking.

The goal of this thesis was to specifically address the following questions:

What is the effect of a specific behavior used as read-out on the estimation of memory formation?

I tested auditory fear conditioning to complex sounds in single mice with two different behavioral readouts, freezing and lick suppression, in order to assess if they influence the sensitivity of the memory tests in different ways.

In order to compare the effect of positive or negative reinforcement of the same neutral auditory stimuli both paradigms need to happen on a similar time scale. Is it possible to design an operant conditioning paradigm involving positive reinforcement that leads to successful learning within a single session, similar as classical fear conditioning paradigms?

For positive reinforcement of a complex sound I designed a modified second-order conditioning learning paradigm using visual and auditory stimuli as salient cues for reward.

Under what behavioral conditions form mice an auditory memory that is specific to the properties of the conditioned sound stimulus?

I established an auditory discrimination task in which one of two complex sounds was positively reinforced. This allowed me to assess the ability of mice to discriminate between complex sounds.

1.2 Material and Methods

1.2.1 Material

1.2.1.1 Experimental set up

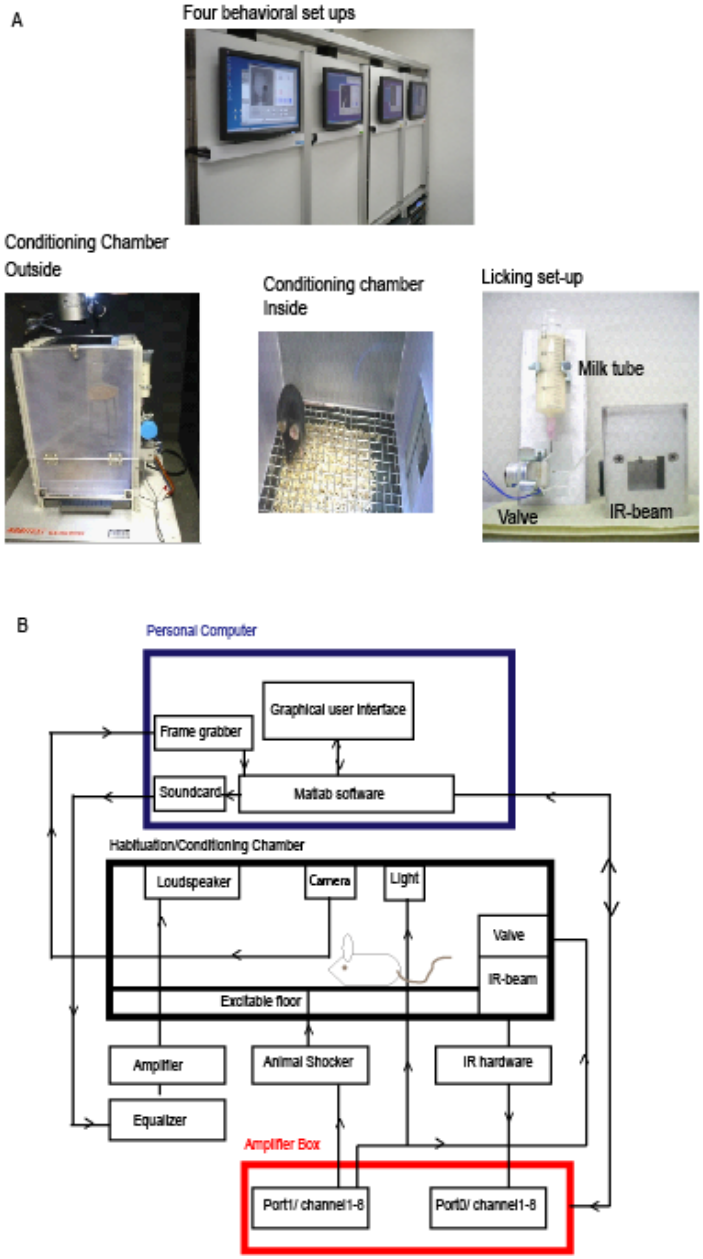


Figure 1 I Experimental set up.

A Pictures of different set-up parts. B Schematic of the conditioning setup.

The setup was replicated identically four times in order to perform the experiments with four mice simultaneously.

PC

MATLAB (Mathworks, Natik, MA) software

Matrix frame generator built in PC

Soundcard for sound application within the range of 4Hz-80000Hz

National instruments digital input and output tower (NI DIO USB6501)

ART Octave Graphic Equalizer (Model #351 31 Band 1/3)

ART SLA1- studio line amplifier

Custom-made loud speakers, frequency range one to 80 kHz

Portable microphone (img stage line HM-30 50 Hz to 20 kHz), stereo microphone amplifier (Monacor MPR-6)

Infrared camera black/white camera with IR-lamp 1/3'' CCD KB-R3138

Ceiling Mount Infrared Activity Detector H24-61

Light source (3 white LEDs)

ITL Pulse generator

Coulbourn Instruments, Lehigh Valley, PA

Coulbourn Precision Animal shocker (from 0 to 3mA)

Coulbourn Conditioning chamber (10x10x17 cm, two oppositely situated metal walls and a third clear transparent wall, model H10-24, modified)

Exchangeable floor (excitable stainless steel bars, model H10-24R and grid floor)

Coulbourn Habitest acoustic isolation cubicle (model H10-24A)

Coulbourn Optical lickometer (model H24-01M & H24-01R)

Coulbourn Single photocell sensor (model H20-94)

Electronic valve, NResearch, 267 Fairfield Ave., W. Calwell, NJ 07006 USA

2 bottom-less cartridges for different environments, a quadrangular clear-transparent plexiglas box with three walls and one opening for the port application and a round white fibreglas tube (diameter 12 cm) with an opening to one side

Metal tube, 2 cm long and 0.2 mm diameter

20 ml syringe, Braun omnifix

Nestle condensed sweetened milk

Voeslauer mineral water without gas

1.2.1.2 Environments

Environment A: Grid floor with bedding of home cage underneath, quadrangular transparent cartridge, licking port, transparent plastic on top, direct light illumination

Environment B: Excitable bar floor with green towel soaked in 70% ethanol underneath, round white fibre glas tube, red plastic on top, direct light illumination.

1.2.1.3 Complex sound stimuli

All stimuli were broadband according to their frequency spectrum and covered parts of the hearing range of the mice. The sounds had a length of two seconds. A CD with a repertoire of natural sounds and different everyday life sounds were used. For all sounds the sampling rate was changed from normal sampling rate for commercial CD of 44000Hz to 91000 Hz in order to broaden the frequency range of sounds to frequencies higher than 20 kHz. All recordings were made at 32bit depth. The loudness was normalized to approximately 70 dB SPL and peak values up to 85 dB SPL with rise and fall times of the sounds were added in order to avoid broadband clicks. (see 3.1. Appendix Sound List)

1.2.1.4 Liquid supply

In preliminary experiments 10% sucrose solution, gatorade and sweetened milk (diluted with water in a ratio of 1:3) were tested for preference (Heffner, Koay et al. 2006; Neubert, King et al. 2008).

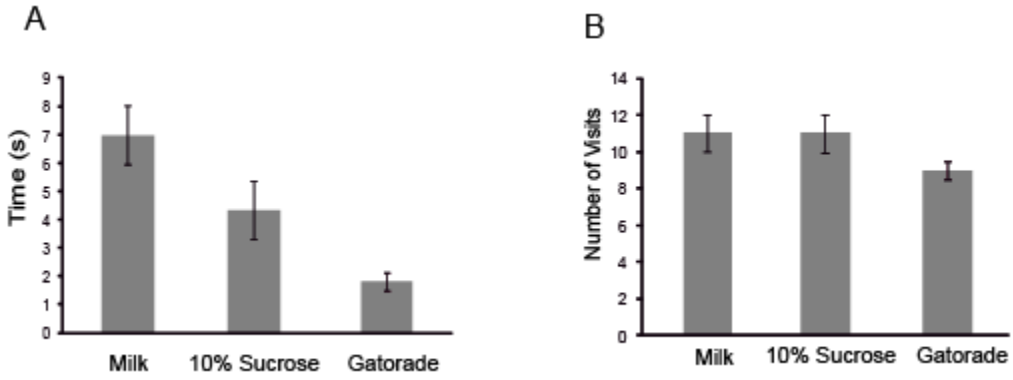


Figure 2 I Testing liquid preference in mice.

Different groups of mice were exposed to three specific liquids, sweetened milk, 10% sucrose and gatorade during test sessions. A Bars represent mean visit duration across mice and sessions, SEM (n=5). B: Bars represent mean number of visits across mice and sessions, SEM (n=5).

In general, mean visit durations for milk were higher than for 10% sucrose and gatorade. Therefore, all experiments were performed with sweetened milk. This indicates that sweetened milk has an intrinsically appetitive value for mice.

In order to control liquid supply a 20 ml syringe was fixed at the outside of the metal wall of the conditioning chamber. It was filled up to maximum with milk. A plastic tube was used to connect the syringe and a short metal tube of 0.2 mm diameter for liquid supply inside the port (mean flow rate: 1.2 ml/min). The plastic tube was additionally inserted into an electronic valve to control liquid delivery. A sensory infrared-beam in the port detected each port entry of the mouse. It was used to open and close the valve.

1.2.1.5 Software

Freezer Software

The custom written MatLab software was programmed by Simon Rumpel. It enabled a user-based control of the essential parameters for classical conditioning by loading different conditioning or memory test protocols. They contained settings for specific parameters, i.e. sound presentation, number of repetitions, number of CS-US parings and shock duration.

Lick software

This custom written MatLab software was programmed by Brice Bathellier and allowed the control of operant conditioning experiments for mice. In particular, liquid delivery in defined temporal relation to sound stimuli was implemented. Additionally, the mouse behavior was online monitored with camera and infrared-beam detection. Different versions of 'Lick' were constructed for specific conditioning paradigms.

Lick2

This program was used for habituation in experiments 3.1.1., 3.2.1., 3.2.2., 3.3., in figure 5, 6, 11 and 14 and allowed control of liquid delivery and display of random background sounds.

Lick3

This program was used for habituation in experiments 3.1.1., in figure 4, 5, 6, and allowed control of liquid and sound delivery after port entry.

Lick7

This program was used for conditioning in experiments 3.2.1., 3.2.2., in figure 10-14 and allowed control of liquid delivery, random representation of blue light flashes and sound delivery before light flashes.

Lick8

This program was used for conditioning in experiment 3.3., in figure 16, 17 and 18 and allowed control of liquid and sound delivery after port entry as well as the setting of time out periods.

1.2.2 Methods

1.2.2.1 Behavioral experiments

All animal experiments described in this work are approved by the Magistratsabteilung 58 of the Wiener Landesregierung, Vienna, Austria in accordance to the §§ 7, 8 10 Abs. 3 Tierschutzgesetz, 1988 (TVG 1988), Austria.

Three to six month old male C47BL/6J wild type mice, commercially obtained from Harlan or in-house bred, were used for all experiments. After weaning on day 21 mice were kept in same sex groups of two to five animals, according to their weight, under normalized conditions with a light-dark circle of 12 hours each, water and food ad libitum. Constant background noise in form of radio broadcasting was present during the light phase of the cycle. All experiments were performed during the light phase of the cycle everyday at the same time.

Habituation

For fear conditioning experiments Lick3-software was started and all setup parameters were checked. Mice were habituated to environment A for five days in two single sessions of 15 min per day. The first one without sound and the second with delivery of one of three background sounds and one blank after port entry. (Heffner, Koay et al. 2006) Mice had free access to milk. On the last three days mice were additionally habituated in environment B for 3 min.

In second order conditioning and discrimination task a milk bottle was exposed in the home cage overnight. The first two days Lick2-software was started and all setup parameters were checked. Mice were put together in groups of five animals in environment A for 60 min in order to allow social learning. They had free access to milk.

Fear conditioning

Freezer software was started, all experimental setup parameters were checked and conditioning protocol was loaded. One complex sound was five times paired with a 0.7 mA foot shock . The sound was delivered at random intervals with intermittent breaks of at least one minute. Shock was delivered 2 seconds after sound delivery.

Fist order conditioning

Lick7- software was started and all experimental setup parameters were checked. Mice were split in two groups with and without randomly displayed background sounds in intervals of 40 to 60 s. Single mice were placed in environment A for 30 min each day. Blue light pulses were delivered in random intervals of 40 to 60 seconds. Each pulse lasted four seconds. Liquid access was restricted to a four seconds opportunity window after light delivery. Milk was delivered in four 0.1 ms pulses always intermitted by a one second interval.

Second order conditioning

Lick7- software was started and all experimental setup parameters were checked. Mice were split in two groups with and without randomly displayed background sounds in intervals of 40 to 60 s. Single mice were placed in environment A for 30 min each day. A complex sound of two seconds duration was delivered in intervals of 40 to 60 seconds. After four seconds time delay a blue light flash was delivered. Liquid access was restricted to an eight seconds opportunity window after sound onset. Milk was delivered in four 0.1 ms pulses always intermitted by a one second interval.

Discrimination conditioning

Lick8- software was started and all experimental setup parameters were checked. Single mice were placed in environment A for 30 min each day. Each port entry initiated the delivery of one of two complex sounds. Two seconds after the positive sound milk was delivered in one 0.1 ms pulse. After the negative sound no milk was delivered.

Memory tests

Three different memory tests were performed 24, 48 hours and on day 15 after fear conditioning.

Lick suppression memory test

Lick2-software was started and all experimental setup parameters were checked. First licking behavior was observed in a 15 min session in environment A without sound delivery. In the

second 15 min session licking behavior was observed after random delivery of conditioned, background or blank sound.

Freezing memory test

After the two lick suppression memory tests Freezer software was started, all experimental setup parameters were checked and memory test protocol was loaded. Freezing levels were measured in a 6-7 min session. Mice were placed in environment A in order to avoid contextual fear responses. In the first minute of the test no sound was delivered in order to examine the general fear response. This is considered as baseline freezing. Then the target sound and two other complex sounds were represented ten times, in two blocks of five stimuli with intermittent intervals of two seconds. The blocks were randomly displayed in intervals of at least 20 s.

Sound Discrimination Test

Complex sound discrimination of mice was tested 24 hours after the last second order conditioning session. Lick7- software was started and all experimental setup parameters were checked. Single mice were placed in environment A for 30 min. The conditioned and three neutral sounds were randomly displayed in 40 to 60 s intervals. No reward was delivered.

1.2.2.2 Analysis of behavioral experiments

Freezer analysis

In order to analyze freezing behavior of mice a custom MatLab software tool was programmed by Simon Rumpel. Raw data of behavioral experiments were saved in MatLab files. Single motion pixel (SMP) differences of consecutive images were calculated. A threshold of 10 SMP's was fixed for freezing detection. SMP levels above or below this threshold were used to calculate percentage freezing.

Lick-analysis

Raw data of the behavioral experiments were saved in MatLab files and analysed with MatLab software.

Metrics

- Correct performance in second order conditioning

Latency is the time elapsing from stimulus onset to port entry. Latencies were computed for all port entries within one session and sorted according to their duration. Latencies shorter than the correct opportunity window of four seconds were considered as correct performance. Mean performance of single mice and across mice was computed for single sessions.

- Estimation of random poking behavior in second order conditioning

The probability of non-rewarded poking was computed by dividing the total number of non-rewarded trials by the total time of the experiment minus the reward delivery time. The probability of poking within a random four seconds opportunity window was computed by multiplying this probability with four seconds. In this computation I assumed mouse behavior as stationary. However, real mouse random poking behavior could vary over time and therefore the expected value could be smaller or greater that the actual computed one. For a better estimation of random non-rewarded poking behavior, generally a blank sound delivery should be included in experiments.

- Correct performance in discrimination task

The infrared beam status was checked in the 500ms after sound offset. For the positive sound at least one beam break in this time window was considered as correct performance. For the negative sound no beam break in this time was considered as correct performance. The total correct performance is the mean of correct performance to positive and negative sound.

Statistical Analysis

One way ANOVA was performed with data of the discrimination test in second order conditioning. P-values less than 0.05 were considered to be statistically significant. A non-parametric Wilcoxon-test was used to examine differences across sound groups.

1.3 Experiments and Results

1.3.1 Testing fear memories in individual mice with different behavioral read outs

The assessment of memory in behavioral experiments always utilizes a specific behavior as readout. For example, freezing behavior is often used in fear conditioning paradigms. However, the specificity and variability of the memory assessment may depend crucially on the particular properties of the behavior that has been used as readout. Here, I was interested in testing the same conditioned mice with two different behavioral readouts to assess potential differences.

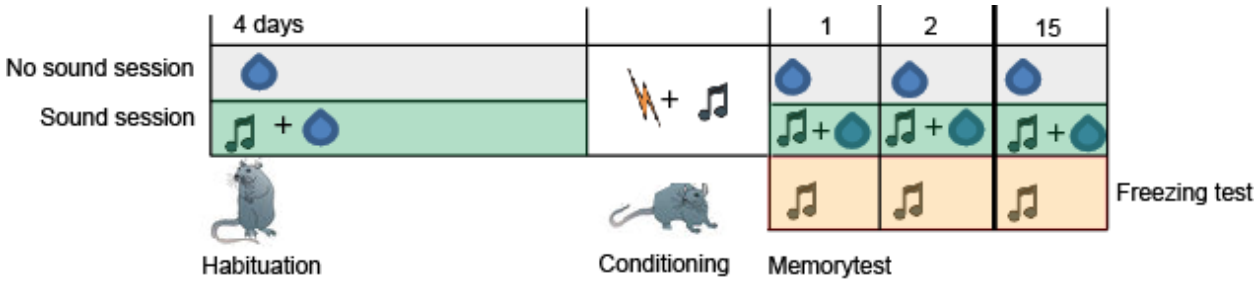


Figure 3 I Fear conditioning.

Experimental time flow.

Mice were habituated as described above for five days (see Habituation 2.2.1.). Briefly, on each day mice experienced two 15 min habituation sessions in which each nose poke was rewarded with sweetened milk. In one session nose pokes only triggered liquid delivery, in the other a sound was presented concomitantly with liquid delivery. On day 6 fear conditioning to the target sound gallop was performed (see Fear Conditioning 2.2.1.): The sound gallop was five times paired with a 0.7 mA foot shock. Following conditioning, three memory tests were performed 1, 2 and 15 days afterwards. On each day mice experienced three consecutive memory test sessions. First, licking behavior was observed in a 15 min session without sound delivery. This allowed me to assess if the process of conditioning would influence mouse licking behavior in a non-specific, non-sound related manner. Second, licking behavior was observed in a 15 min session with presentation of the target sound gallop, two background sounds, beet and keep, or a blank

after port entry. Each of the four stimuli was played in random order. This allowed me to test if the mouse had learned the association between target sound and foot shock as indicated by suppressed licking when the sound was played. Third, freezing levels were measured as described above to the target sound gallop and the two background sounds beet and keep (see Memory Test Freezing 2.2.1.). The assessment of freezing behavior was done in an experimental setup that did not involve the mice in licking behavior. Playing the conditioned sound as well as non-conditioned sounds allowed us to estimate the degree of generalization.

1.3.1.1 Assessing memory formation using lick suppression

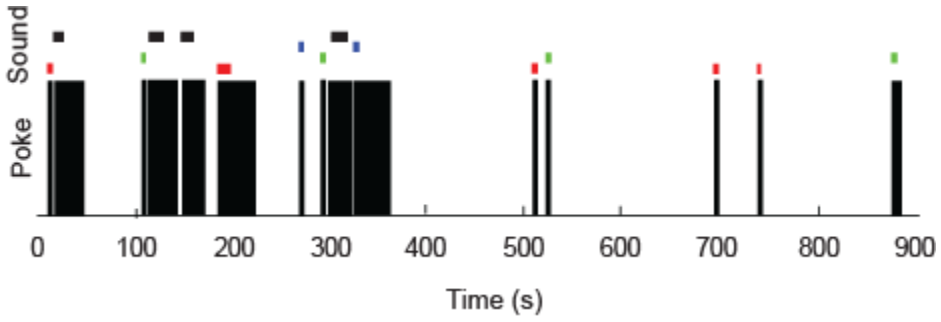


Figure 4 I Single mouse licking performance in a typical habituation session with presentation of different sounds.

This is an example for licking behavior of one mouse during a 15 min session which was habituated to sound presentation concomitant with liquid delivery before conditioning. Black bars represent poke duration, colored bars above indicate the presentation of four different sounds, red gallop, blue beet, green keep and black blank.

During the first days of habituation I observed that mice showed shorter visits when a sound was presented as compared to blank or no sound session. This difference became smaller with additional habituation. Nevertheless, even before conditioning average visits to different sounds were typically shorter than to blank and were to some extent different to each other, but provided us with a measure of average licking behavior that is associated to a particular sound. Based on previous studies, a dramatic shortening or suppression of the licking would be expected if the mice would show fear (Vanover, Robledo et al. 1999; Heffner, Koay et al. 2006; Neubert, King et al. 2008).

As previously mentioned, I needed to habituate mice to sound presentation concomitant to liquid delivery for several sessions. The graphs represent data corresponding to the last four days of habituation in which average licking behavior to sounds reached 5-10 seconds. I aimed for

average visit durations above 4 seconds in order to allow the assessment of shortening or suppression of licking after conditioning. Nevertheless, sometimes few mice showed generally low licking behavior in a session, so that it was impossible to obtain lick suppression data for all of the sounds. This explains the variable ‘n’ in the following analysis (see Fig. 5).

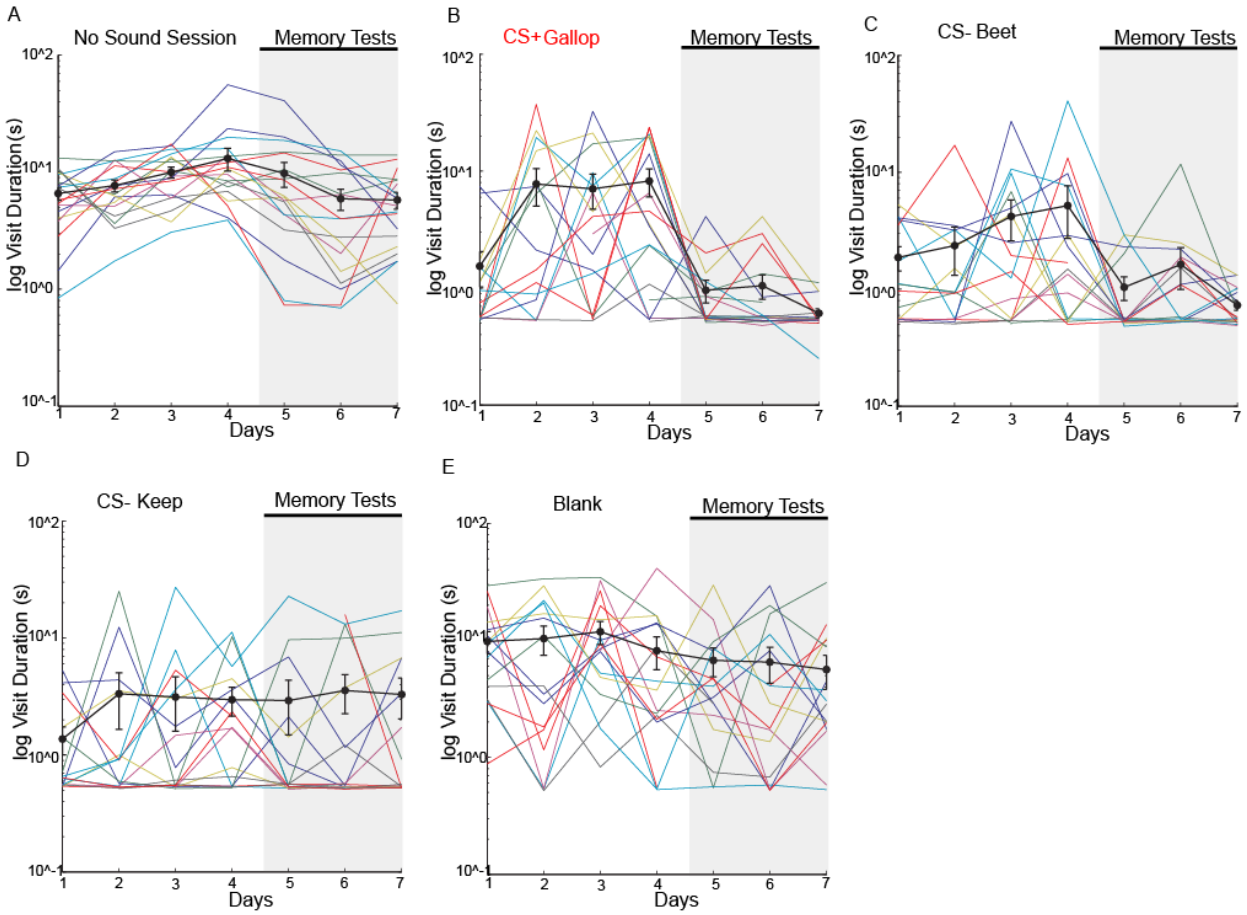


Figure 5 I Licking behavior before and after fear conditioning to sound gallop.

Licking behavior is indicated by mean visit duration of single mice over sessions. Few mice only rarely visited the port. Therefore, the total number of mice per day varies between 16-18 which is indicated specifically for each graph. In this experiment the sampling rate for nose pokes and thus also the minimal visit duration was 500 ms. A No sound session, colored lines represent mean visit duration of single mice over sessions, thick black line represents mean visit duration across mice over sessions, grey area represents memory test sessions on day 1, 2 and 15 after conditioning, SEM (n=18). B Sound session gallop, as described in A, SEM (n=18, except day1/17, day2/16, day3/16, day7/16). C Sound session beet, as described in A, SEM (n=18, except day1/16, day2/16, day4/17, day5/16, day7/17. D Sound session keep, as described in A, SEM (n=18, except day1/17, day2/16, day4/17, day5/17, day7/16). E Sound session blank, as described in A, (n=18, except day1/17, day2/16, day3/17, day4/17, day5/17, day6/16)

First, I wanted to assess if the process of conditioning would alter licking behavior of mice in a non-specific, non-sound related manner. After conditioning I observed that mean visit durations did not change dramatically compared to pre-conditioning sessions. On average visits lasted 8-10 seconds before and after conditioning. Furthermore, licking behavior after presentation of blank decreased only little after conditioning indicating that shock delivery did not influence licking behavior. In daily 15 min sessions mice were habituated to sound presentations concomitant to liquid delivery. Therefore, after conditioning to gallop visit durations should decrease in order to indicate lick suppression. One day after conditioning I observed decreases of visit durations below 4 seconds after presentation of gallop representing lick suppression. However, visit durations after presentation of beet decreased similarly indicating generalization. In contrast, licking behavior after presentation of keep was not affected. However, visits specific to this sound were already short during habituation indicating that keep could have been intrinsically fearful. Nevertheless, this result showed also that mice were able to discriminate between sounds. I tested long-term memory formation on day 2 and 15 after fear conditioning. Mice showed lick suppression responses similar to memory tests one day after conditioning. Thus, a robust association between sound and shock had been formed. Furthermore, I could not observe latent inhibition of the sound gallop which would mean that learning performance could be decreased by pre-presentation of the conditioned sound in an unpaired fashion (Barad, Blouin et al. 2004).

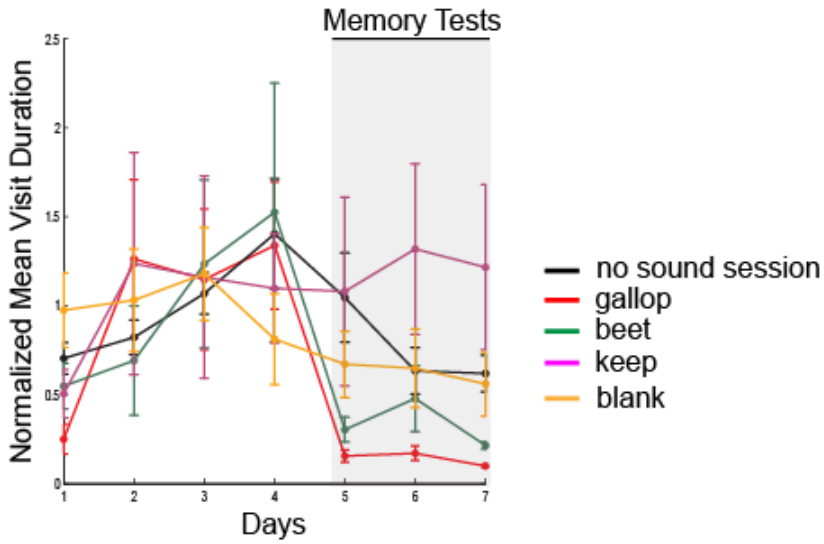


Figure 6 I Mean normalized licking behavior before and after conditioning to gallop.

Mean visit durations across mice were normalized to baseline licking which represented the total mean visit duration across mice over the first four days. Colored lines represent normalized mean visit durations across mice over sessions corresponding to sound display, black no sound session, red sound session gallop, green sound session beet, violet sound session keep, orange sound session blank, grey area represents memory tests on day 1, 2 and 15 after conditioning.

During habituation I observed that sounds influenced mouse licking behavior in different ways. For example, visits after presentation of the sound keep were in general shorter compared to gallop and beet. Therefore, the effect of conditioning to gallop could be occluded by intrinsic sound properties. In order to discriminate between conditioning and sound induced effects on licking behavior we normalized mean visit durations to baseline licking presented by the total mean visit duration across mice over the first 4 days of habituation. After normalization we still observed high generalization for the sounds gallop and beet. However, licking after presentation of keep was not affected after conditioning indicating that this sound was intrinsically fearful. Visit durations in the no sound session and after blank presentation slightly decreased indicating non-sound related effects of conditioning on licking behavior.

1.3.1.2 Assessing memory formation using freezing behavior

After the two lick suppression tests I additionally performed with the same mice a freezing memory test to asses if the two behavioral read outs would show potential differences.

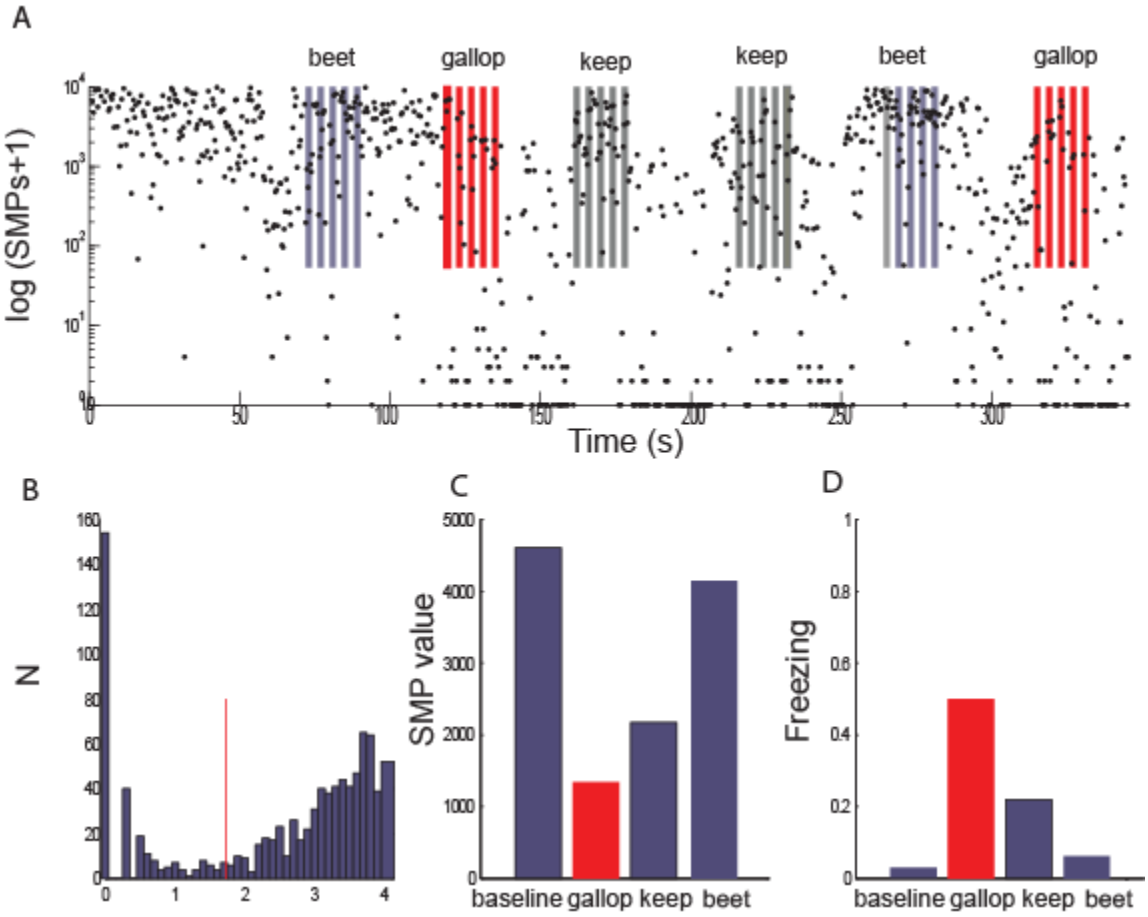


Figure 7 I **Single mouse freezing behavior in a typical memory test after conditioning to gallop.**

This is an example of a mouse which showed significant freezing to the target sound gallop. SMP values were low during sound display of gallop indicating few or no motion of the mouse. This was considered as freezing behavior. A Single SMP values for stimuli and baseline. B: Distribution of SMP values. C Mean SMP values for stimuli and baseline. D Percentage freezing (SMP<53.2785) for stimuli and baseline.

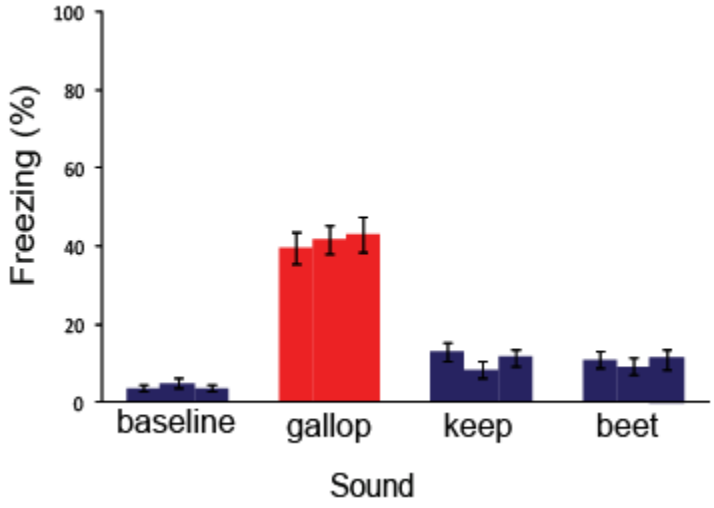


Figure 8 I Mean freezing behavior in three memory tests after conditioning to gallop.

Bars represent mean freezing levels across mice in three different memory tests, from left to right 1, 2 and 15 days after conditioning, red bar represents conditioned sound gallop SEM (n=7).

Mice showed low baseline freezing levels indicating no contextual fear responses. Furthermore, I observed robust freezing to the target sound gallop with 50% freezing levels. Freezing to gallop was higher than to back ground sounds beet and keep indicating discrimination of complex sounds. Freezing levels did not change in memory tests on day two and 15 after conditioning, thus, a robust memory was formed. Additionally, I did not observe extinction which means that a learned association would be lost due to unpaired stimulus representations after conditioning (Bouton, Westbrook et al. 2006).

In summary, both memory read outs, lick suppression and freezing, showed high sensitivity to conditioning to the target sound. Interestingly, generalization of fear-related behavior in the freezing test appeared more specific and showed less generalization than estimated from lick suppression.

1.3.2 Using second order conditioning to accelerate positive reinforcement paradigms

The aim of this experiment was to establish a robust and fast learning paradigm for positive reinforcement of complex sounds in mice. In general, operant conditioning is a time consuming process since it relies on the amplification of initial random mouse behavior. Typically, during operant conditioning the mouse has to learn several procedural aspects of the task which go beyond the simple stimulus→reward association. This is likely the reason for the extended training necessary in operant conditioning. Therefore, I split up the learning process in two phases which is reminiscent of classical second order paradigms. In classical second order conditioning pairing of CS1 and CS2 occurs without reward delivery. However, in my paradigm CS1 and CS2 were both paired with reward for reasons explained below. Despite this difference to classical second order paradigms I kept the terminology of 'first order conditioning' and 'second order conditioning'. In my paradigm mice learned during first order conditioning that a salient light stimulus predicted the availability of reward. In second order conditioning light was preceded by a complex sound as predictor for light and thus reward. With this strategy I wished to establish a salient, positive cue, i.e. light, that could then be quickly utilized for association with a neutral sound stimulus, analogously to a foot shock. In classical second order paradigms extinction can occur when the animal learns that CS1 no longer predicts the US (Gewirtz and Davis 2000). In order to prevent this I decided to keep the contingency between sound→light→reward and sweetened milk was available during both CS1 and CS2.

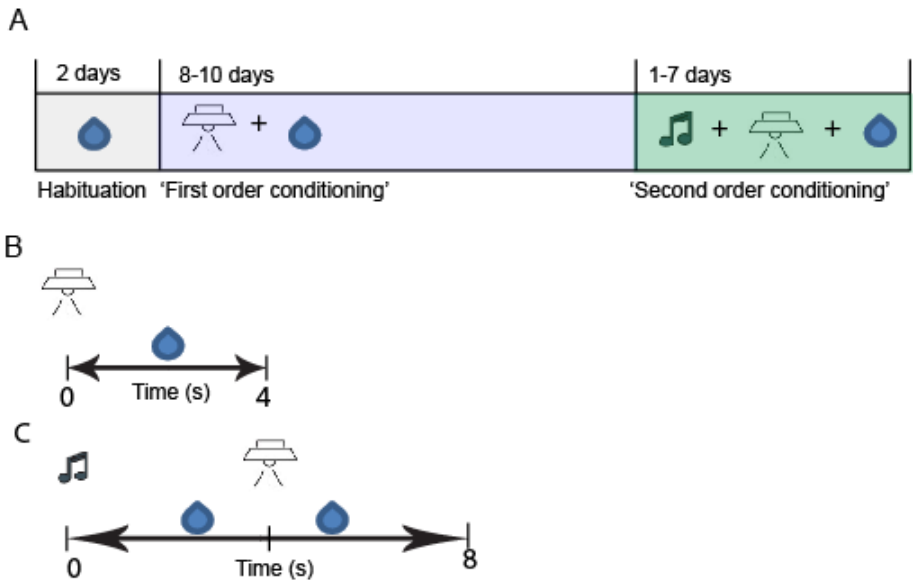


Figure 9 I Second order conditioning.

A Experimental time flow. B First order conditioning phase, reward is available in a 4 s time window after light onset. C Second order conditioning phase, reward is available in an 8 s time window after sound onset.

Mice were habituated and familiarized to the drinking spout for two days. Each nose poke was rewarded with sweetened milk (see Habituation 2.2.1.). First order conditioning to light was performed for 8 to 10 days (see First order conditioning 2.2.1.). During this period mice learned that liquid reward was only available in a four seconds time window after light presentation. Finally, second order conditioning was performed to the target sound beet (see Second order conditioning 2.2.1.). Reward was now available in an eight seconds time window after sound presentation.

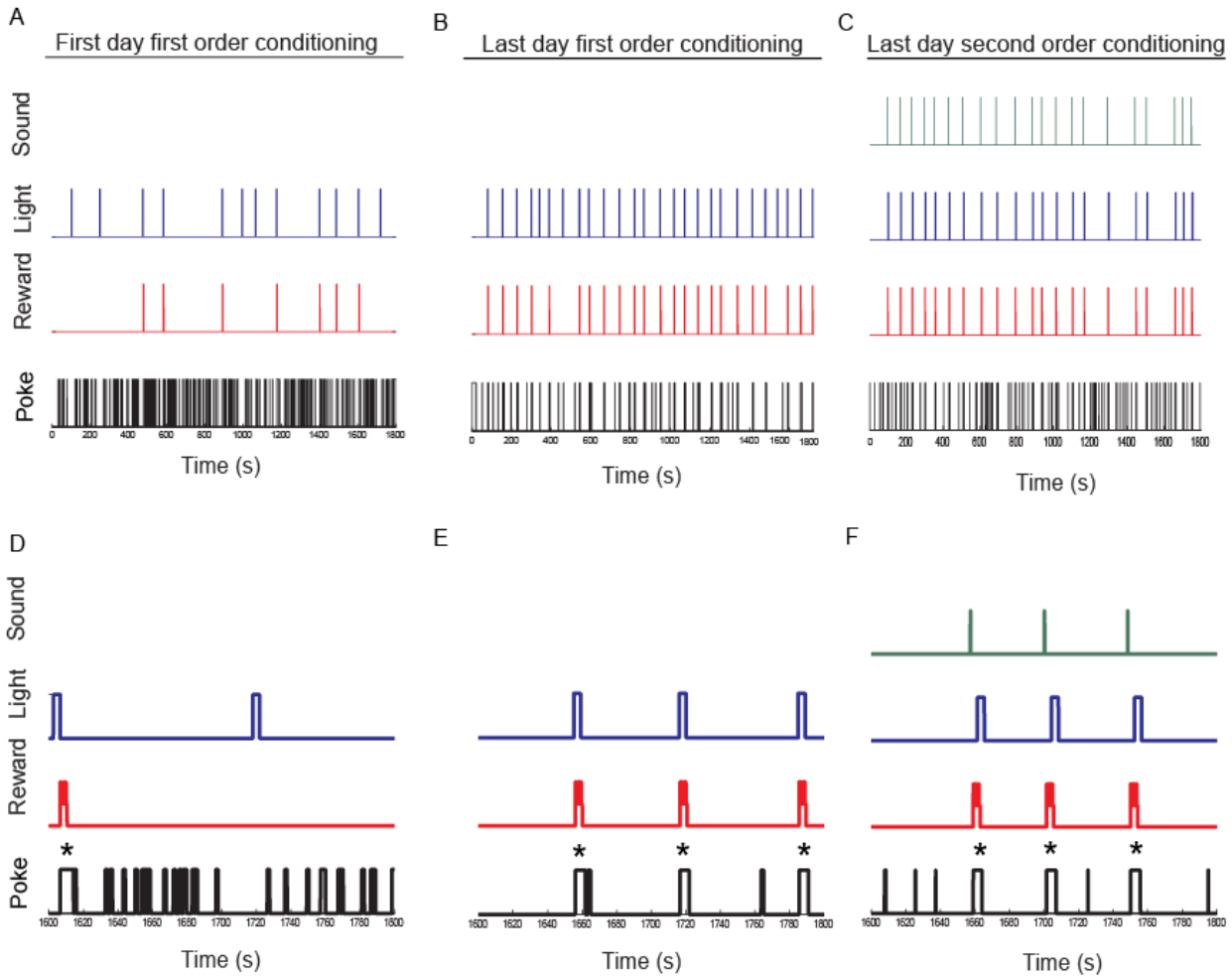


Figure 10 I Single mouse licking performance at three time points in second order conditioning.

A First session of first order conditioning, nose pokes of the naive mouse are not correlated to light presentation, thus, few rewards are delivered, black bars represent individual pokes, red bars represent reward delivery, blue bars represent light delivery. B Last session of first order conditioning, nose pokes of the trained mouse correlate strongly to light delivery, almost every poke is rewarded C Last session of second order conditioning, nose pokes of the trained mouse correlate strongly to sound onset, almost every poke is rewarded, green bars represent sound delivery. D Enlarged 200 s of conditioning session presented in A. E: Enlarged 200 s of conditioning session presented in B, asterisks indicate rewarded pokes F: Enlarged 200 s of conditioning session presented in D, asterisks indicate rewarded pokes.

Figure 10 shows an example for a single mouse performance at three different time points in second order conditioning. During the first phase of first order conditioning mice had to learn that sweetened milk was only available in a 4 second time window after light presentation. Therefore, in the beginning only few port entries of the naive mouse occurred in the correct time

window and were rewarded. After consecutive days of conditioning to light mice learned that light predicted reward and almost every port entry was rewarded. Furthermore, the initial high unspecific poking rate decreased, indicating learning. When mice took light as salient predictor for reward I started second order conditioning to the sound beet. Once the sound was associated with the first stimulus light it became a salient predictor for reward. In the last second order conditioning session almost every rewarded poke corresponded rather to sound than to light onset and was rewarded.

1.3.2.1 Positive reinforcement of complex sounds using multiple second order conditioning sessions

In classical second order conditioning, typically an auditory or olfactory stimulus is paired with a mild foot shock and freezing or potentiated fear startle are used as memory read out (Gewirtz and Davis 2000; Paschall and Davis 2002; Debiec, Doyere et al. 2006). However, I wanted to test second order conditioning with positive reinforcement of visual and auditory stimuli. Initially, light was rewarded with sweetened milk. Then, during second order conditioning a sound was introduced with 4 seconds delay to light onset. Therefore, if mice learn the association between light and sound as predictor for reward they enter the port rather after sound than light onset. I was interested to know how long first order conditioning to light would last since this phase includes learning procedural aspects of the task. Furthermore, I wanted to assess how fast mice would associate the presented sound with light and subsequently use it as predictor for reward. Additionally, I wanted also to examine if learning and behavioral changes occur progressively or in an all-or-nothing manner. In the final session, after second order conditioning, I tested discrimination of sounds in order to examine potential differences to fear conditioning.

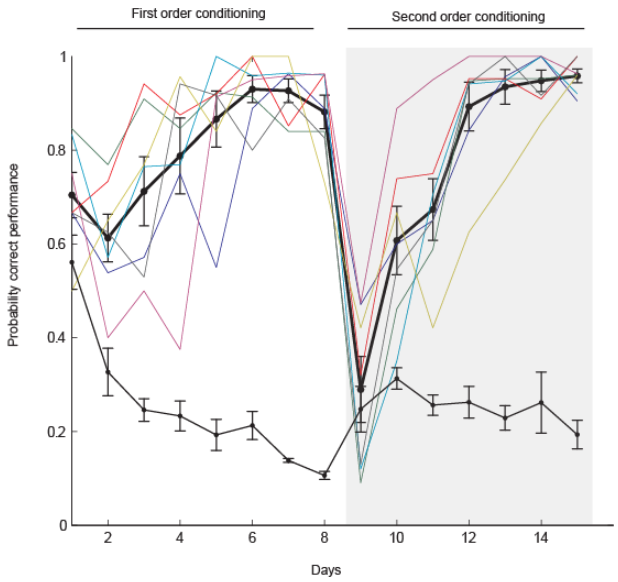


Figure 11 I Learning curve of first and second order conditioning to light and sound.

Correct performance of mice was assessed by counting only port entries occurring in the correct time window after stimulus onset. First mice were conditioned to light and afterwards light and sound predicted reward represented by the grey area. Colored lines represent mean probability of correct performance of single mice and sessions, thick black line represents mean probability of correct performance across mice and sessions, thin black line represents estimated random poking behavior, SEM, (n=7). Note, after initial improvement during first order conditioning, performance drops and quickly recovers when switching to second order conditioning.

The presented data is from first order conditioning with the following protocol parameters: light duration and opportunity widow as indicated in Fig. 9, and a 2sec/four pulse reward. However, before that the mice were temporarily exposed to first order conditioning with less stringent parameters. Therefore, correct performance levels started at relatively high levels of 60-70%. I used correct performance levels of mice as behavioral read out. In first order conditioning every port entry within the first 4 seconds after light was considered correct. After eight days, mice showed stationary correct performance of 90% indicating robust learning. Therefore, I started second order conditioning to the target sound beet in which visits in the first 4 seconds after sound onset were considered correct. As expected, in the first session average correct performance dropped near to estimated random poking level since mice still took light as predictor for reward and visits occurred after light onset. However, single mice already showed performance levels above chance indicating that they responded to light and sound. Within few sessions mice learned that sound was a salient predictor for reward and performance levels reached again 90%.

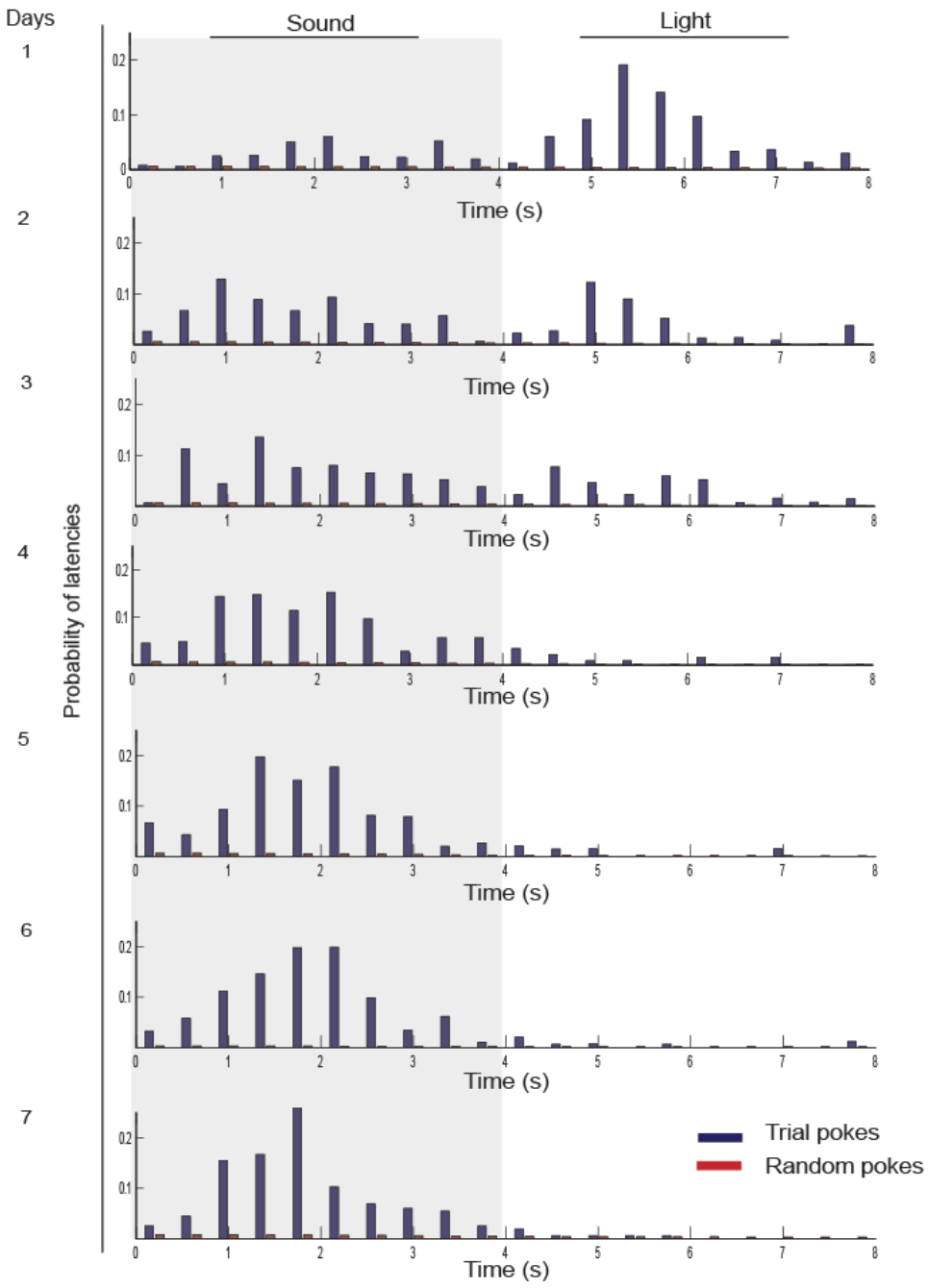


Figure 12 I Learning during second order conditioning expressed in latencies of port entries after sound onset.

Latency is the time elapsed between stimulus onset and port entry. Shown are mean probability of latencies across mice (n=7). Panels 1-7 represent consecutive days of second order conditioning to the sound beet, blue bars represent probability of latencies with respect to the time axis of 8 seconds, red bars indicate estimated random poking behavior, grey area represents correct 4s time window after sound onset. Note, mice show a shift in mean latencies towards the sound onset, indicating the association of sound as predictor of reward.

I wanted to examine if learning and behavioral changes occur progressively or in all-or-nothing dependent manner and used latencies of visits as behavioral read out. Latency is the time elapsed between stimulus onset and port entry of a mouse. Therefore, if mice learn that sound predicts reward, latencies correspond to sound onset and lie between 0 and 4 seconds. In contrast, if latencies correspond to light onset they lie between 4 to 8 seconds. As expected, in the first second order conditioning session most latencies were still distributed around 4 to 8 seconds since mice responded to light as salient predictor for reward. However, within the next two sessions mice began to enter the port after sound and light onset. Latencies now were distributed in a distinctive, bi-modal manner around both stimuli onsets.

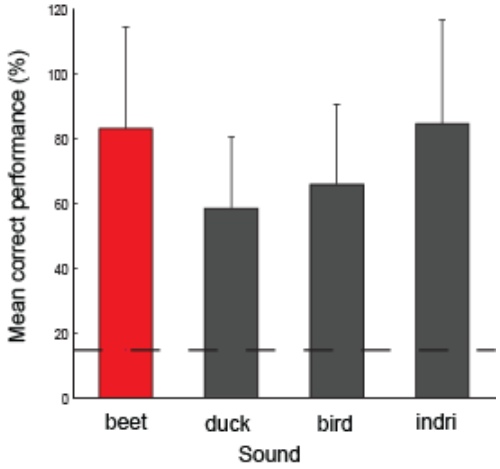


Figure 13 I Discrimination of complex sounds after positive reinforcement.

After second order conditioning, discrimination of four complex sounds without reward delivery was tested. Visits in the 4 s time window after sound onset were considered correct, bars represent mean correct performance across mice, red bar represents the target sound gallop, grey bars represent neutral sounds duck, bird, indri, dotted line indicates estimated random poking behavior, SEM, (n=7). Note, that mice show conditioned behavior to sounds, however, also show broad generalization.

I wanted to examine potential differences in discrimination of complex sounds after positive and negative reinforcement. In the test session the target sound gallop and three different neutral and novel sounds (duck, bird and indri) were presented without reward delivery. This allowed me to assess conditioning effects specific to sound presentation rather than to reward delivery. I observed that mice visited the port after each sound onset regardless if the target sound beet or one of the neutral sounds was presented. Therefore, generalization of complex sounds was high after positive reinforcement. Furthermore, mice showed robust learning of the auditory-light cue

association. For all sounds probability-values computed in a non-parametric single paired Wilcoxon statistical test were significantly different from estimated random poking ($p < 0.05$).

1.3.2.2 Positive reinforcement of complex sounds using one second order conditioning session

The previous experiment showed that mice show almost complete shifting of response latencies within four second order conditioning sessions in which sound predicted the reward. However, in the first session some mice already started responding to sound as salient predictor for reward. Therefore, I considered that learning already starts before the full shift in behavior is completed. In order to answer this question I tested second order conditioning to the same complex sound in one prolonged 60 min session. Furthermore, this would accelerate our learning paradigm. I considered also that presentation of background sounds during first order conditioning could improve learning of a specific light-sound association. Therefore, I tested two groups of mice with and without presentation of background sounds during first order conditioning.

I habituated mice and performed first order conditioning similar to the previous experiment. Mice of one group were habituated and conditioned underwent first order conditioning without background sounds, whereas the mice of the other group underwent first order conditioning with background sounds indoor, bird and duck randomly presented in 40 to 60 s intervals. After seven days mice reached constant high performance levels and we tested second order conditioning to the sound beet in one single 60 min session instead of multiple 30 min sessions as in the previous experiment. Finally, after second order conditioning discrimination of complex sounds was tested with the target sound beet, the background sound duck and two neutral sounds keep and indri.

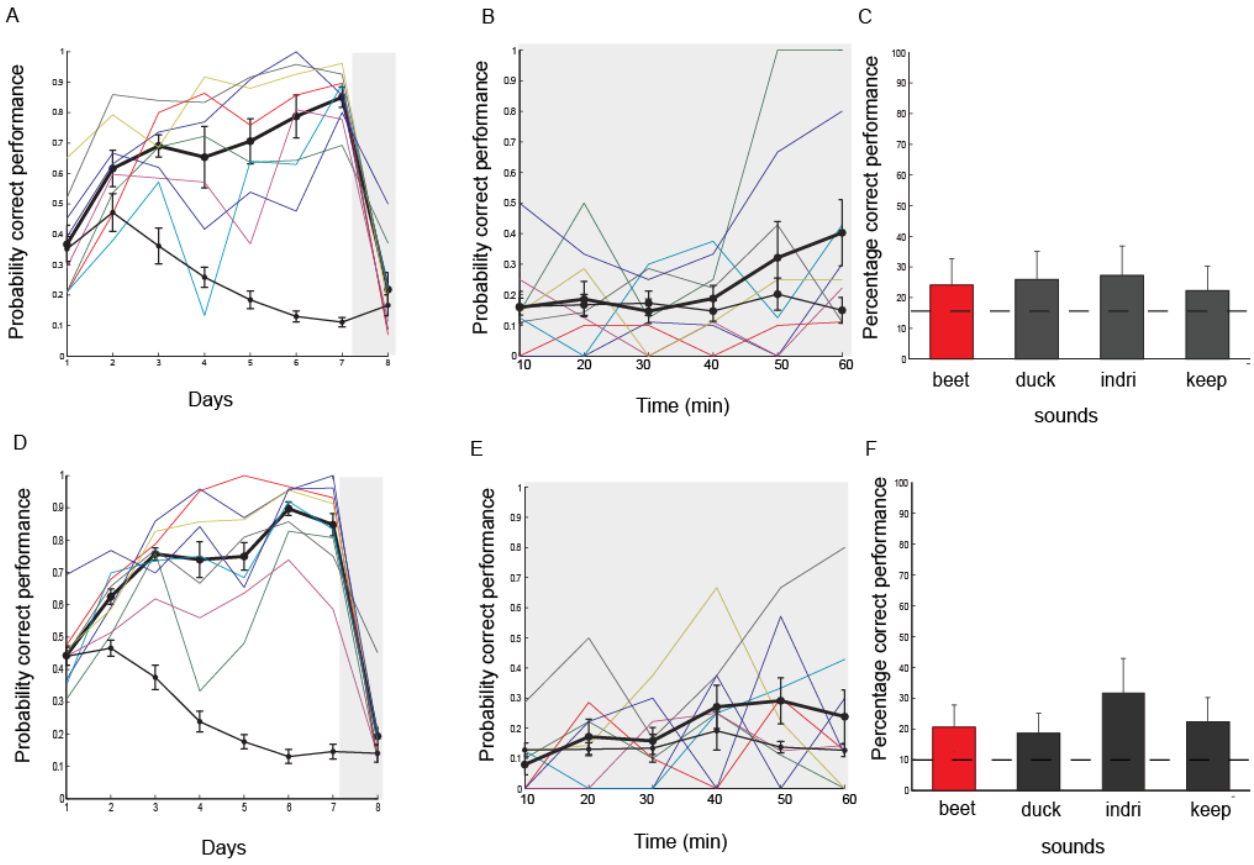


Figure 14 I First order conditioning to light and second order conditioning to a complex sound in one single session.

Correct performance of mice was assessed by counting only port entries occurring in the correct time window after stimulus onset. First, mice were conditioned to light and afterwards light and sound predicted reward, represented by the grey area. A Mice conditioned to background sounds, colored lines represent mean probability of correct performance of single mice and sessions, thick black line represents mean probability of correct performance across mice and sessions, thin black line represents estimated random poking behavior, SEM, (n=8). B: Enlarged 60 min session of day 8 presented in A, as described in A C: Mean correct discrimination performance of mice conditioned without background sounds, as described in A SEM, (n=8), D mice conditioned without background sounds, as described in B, E Enlarged 60 min session of day 8 presented in D, as described in A, F Mice conditioned without background sounds, as described in C.

Within seven first order conditioning sessions mice learned that light predicted reward and both groups reached high probability of correct performance. Behavior was similar in both groups indicating that the presentation of background sounds did not alter learning during first order conditioning. After mice reached constant correct performance we tested second order conditioning to beet within one 60 minute session. I analyzed correct performance within ten

minute intervals in which every port entry within 4 seconds after sound onset was considered correct. During the first 30 minutes correct performance dropped to estimated random poking level. However, in the second half of the session mice started to take both stimuli as predictor for reward indicated by single performance levels above estimated random poking. Performance was similar in both groups indicating that presentation of background sounds did not alter learning during second order conditioning. Finally, on the next day I tested discrimination performance in one 30 min session and presented the target sound beet, one habituation sound duck and two neutral sounds indri and keep randomly without reward. Mice entered the port after all four sound onsets with similar probabilities tested in one-way ANOVA statistical test (group 1/$p=0.9$, group2/$p=0.6$). The performance of both groups was similar indicating that conditioning to background sounds did not improve discrimination. However, compared to the previous experiment discrimination performance was very low. Despite the fact that average performance was higher than estimated random poking, it was *not* significantly different when tested in single-paired Wilcoxon statistical tests ($p>0.05$). This indicated that the initial learning observed during the second order session the day before did not establish a sufficiently strong association between sound and light that could be robustly detected.

In general, I find that initial learning of a positively reinforced sound can occur in a single conditioning session using a modified second order paradigm, however, the performance increase is still rather low. Possibly, adjustments of reward parameters could in the future lead to further improvements to this respect.

1.3.3 Testing discrimination of complex sounds in mice using an operant go/no-go conditioning paradigm

Neural mechanisms of sensory information processing and discrimination of stimuli are critically dependent on time and similarity of stimuli. Therefore, reaction times of responses to given stimuli are commonly used as behavioral read-out for learning. Typically, this is assessed in operant go/no-go learning paradigms. Two different stimuli are either rewarded (S+) or not (S-) and animals need to discriminate between them in order to receive reward. So far, most studies in mice focused on information processing in the olfactory bulb (Abraham, Spors et al. 2004; Rinberg, Koulakov et al. 2006). Abraham et al. for example designed a go/no-go task in which mice needed to discriminate between odors of different complexity in order to receive reward. Since little is known about processing of complex auditory stimuli in mice I decided to test discrimination using a go/no-task similar to Abraham et al. In principle, in my task olfactory cues were replaced by auditory complex stimuli. Discrimination performance was assessed using the probability of correct responses during a given test session.

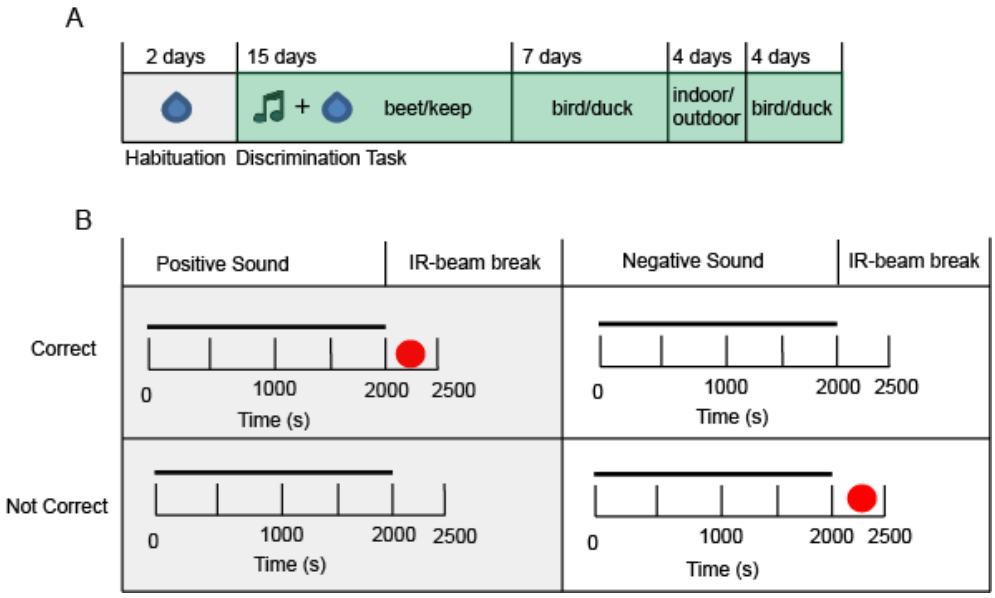


Figure 15 I Discrimination task.

A Experimental time flow. B Assessment of correct performance. After positive sound presentation mice should lick continuously and an IR-beam 2.5 s after sound onset was considered correct, after negative sound presentation mice should leave the port and no IR-beam-break 2.5 s after sound onset was considered correct.

Mice were habituated and familiarized to the drinking spout for two days. Each nose poke was rewarded with sweetened milk (see Habituation 2.2.1.). Then discrimination conditioning was performed with specific sound pairs in which only the positive sound was rewarded (see Discrimination Conditioning 2.2.1.). In order to control for a possible bias by specific sound preferences each sound pair was tested with inverse rewarding values in two groups of mice. For example, during conditioning to the sound pair beet/keep, mice of one group received reward after presentation of beet and the other one after presentation of keep. Furthermore, I considered that neural computations underlying discrimination of two complex sounds possibly involve information about both stimuli. To control for possible effects specific to the combination of two sounds, we tested three different sound pairs within the total experiment. First, the sound pair beet/keep was presented 15 days and consecutively I introduced the new sound pair bird/duck. After 7 days the third sound pair indoor/out was introduced. In order to investigate long-term memory formation of positive reinforced sounds I introduced again bird/duck after 4 days. Learning was assessed through classification of performance into correct and not correct trials. After positive sound presentation mice needed to lick continuously in order to perform correct and receive reward. Therefore, I tested the mouse presence in the port 2.5 seconds after sound onset which would be indicated by an IR-beam break. After presentation of the negative sound mice should leave the port since no reward would be delivered. Thus, the absence of an IR-beam break 2.5 s after negative sound onset was also considered correct. During initial conditioning I observed high numbers of nose pokes. This made the assessment of learning performance of mice difficult since, in principal, high poking rates could be present a behavioral strategy to receive maximal amounts of reward without attention to any sound. Therefore, I implemented a time out penalty of 30 seconds after not-correct performance in which mice could not initiate a trial and no sound was delivered after port entry. This should reduce high poking numbers and force the mice to perform the task in a correct manner.

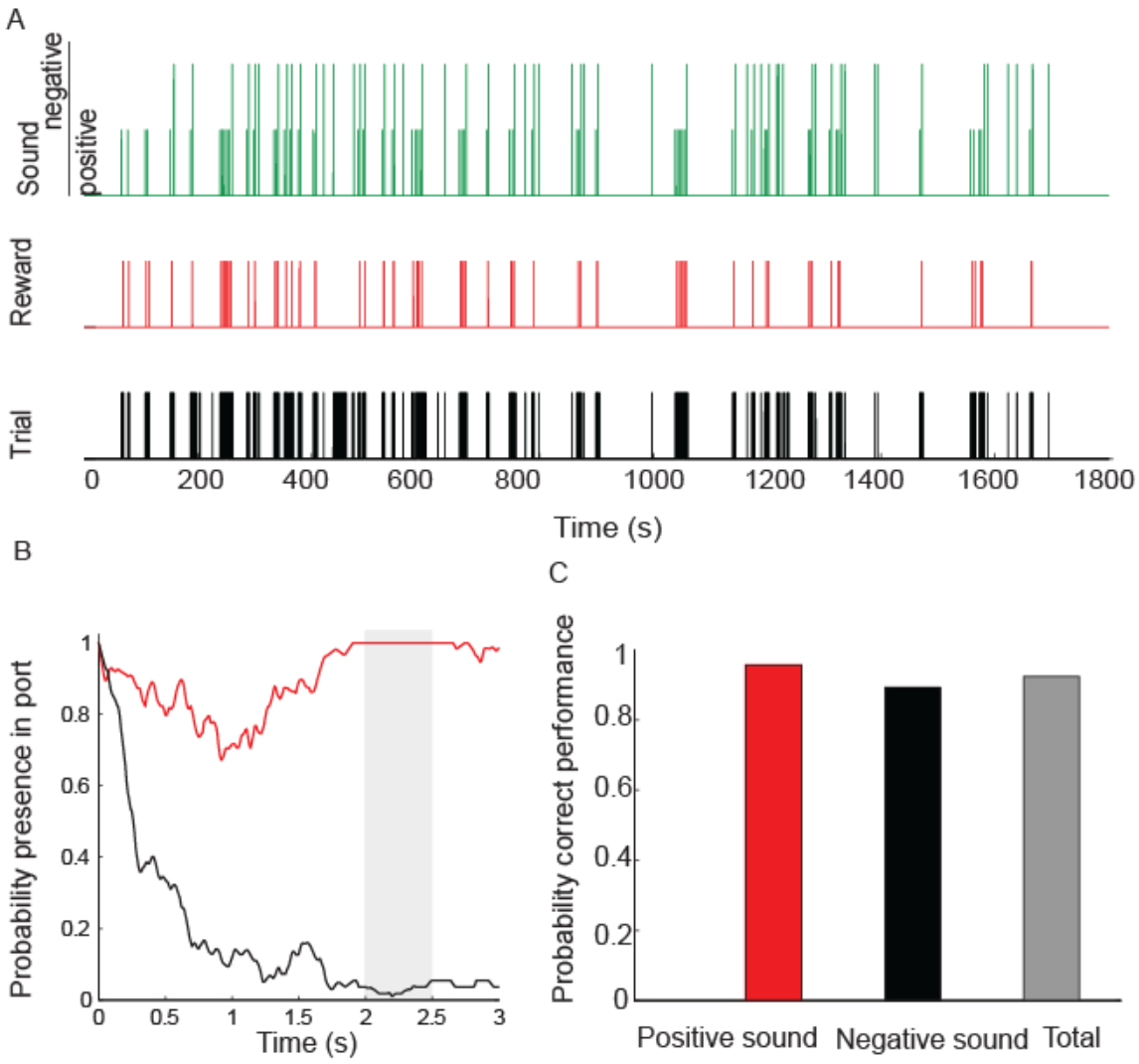


Figure 16 I Performance of a good performing single mouse in a conditioning session after several days of training.

A Example of a trained mouse which shows correct performance and receives high number of rewards. Pokes are presented in relation to reward and sound delivery. Black bars represent individual pokes, red bars represent reward delivery, short green bars represent positive sound delivery, long green bars represent negative sound delivery. B Probability of mouse presence in port within the first three seconds after sound onset, red curve represents positive sound, black curve represents negative sound. C Probability of correct performance presented in B, red bar represents positive sound, black bar represents negative sound, grey bar represents total correct performance.

Figure 16 shows an example of a trained mouse displaying high levels of correct performance. Most positive sounds were rewarded. Furthermore, few time out penalties occurred which would be indicated by long breaks without trials. In order to assess discrimination performance more detailed, I computed the probability of mouse presence in port during three seconds after positive and negative sound onset. Basically, mice could either leave or stay in the port within this time period. As expected from performance presented in 16 A, probability curves in 16 B differed for each sound. Probability of presence in port was high after positive sound presentation in contrast to the negative sound. Since mice could perform for both sounds in a correct manner, the total correct performance represents the mean of both values.

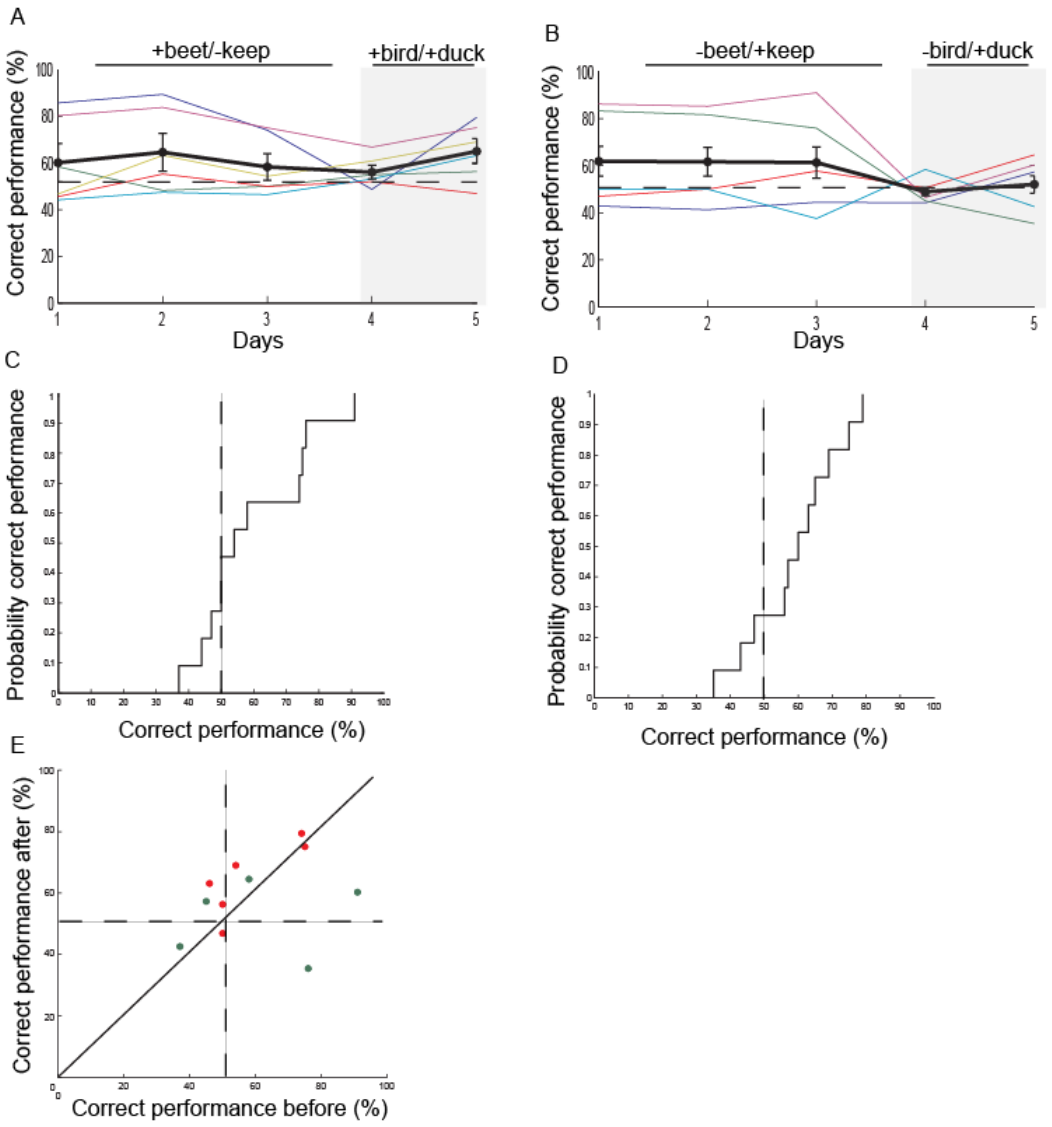


Figure 17 I Discrimination performance during switch of the sound pairs beet/keep and bird/duck.

A One group of mice received reward after presentation of beet and bird, colored lines represent mean correct performance of single mice and sessions, thick black line represents mean correct performance across mice and sessions, dotted black line represents chance level, grey area represents test of the sound pair bird/duck SEM (n=6). B The second group of mice received reward after presentation of keep and duck, as described in A (n=5). C: Cumulative probability of mean correct performance across mice to the sound pair beet/keep on day 3 (n=11). D as described in C, on day 5 conditioning was performed to the sound pair bird/duck (n=11). E Correlation of correct performance of day 3 and 5, red dots represent single mouse performance of group one with positive reinforcement of beet and bird (n=6), black dots represent single mouse performance of the second group with positive reinforcement of keep and duck (n=5).

In order to prevent bias by sound preferences I tested each sound pair with inverse rewarding values in two groups of mice. For example, during conditioning to the sound pair beet/keep mice of the first group received reward after beet and in the second group after keep. However, during the first conditioning to beet/keep I observed similar performance levels for both groups indicating that both sounds were similarly used after positive and negative reinforcement. Generally, I observed that correct performance levels were on average just above chance. Only four out of eleven mice (~36%) showed high learning performance between 80-90%. After 15 days of conditioning to the sound pair beet/keep I introduced the new sound pair bird/duck again with inverse predictive values for both groups. On average performance levels did not change and were similar to conditioning to beet/keep (Fig. 17 A,B). Furthermore, performance of both groups was similar indicating no sound preferences. Additionally, I was interested to test the variability of individual behavior across the total mouse population. I computed cumulative probabilities of correct performance for the day before and after the sound pair switch (Fig. 17 C, D). Typically, if mice learn the task probabilities of correct performance should be shifted towards values above 50% performance. However, I observed that most probabilities were close to the chance level and variation across the population was low indicating that most mice did not acquire the task efficiently. In order to test how sound pair composition influences the discrimination performance of single mice I correlated performance before and after the sound pair switch. Theoretically, if sound pair composition would not influence discrimination behavior of mice, performance before and after the sound pair switch should correlate strongly. However, I observed that most mice performed at chance levels before and after the sound pair switch, making it difficult to detect possible correlations. This was similar for both groups.

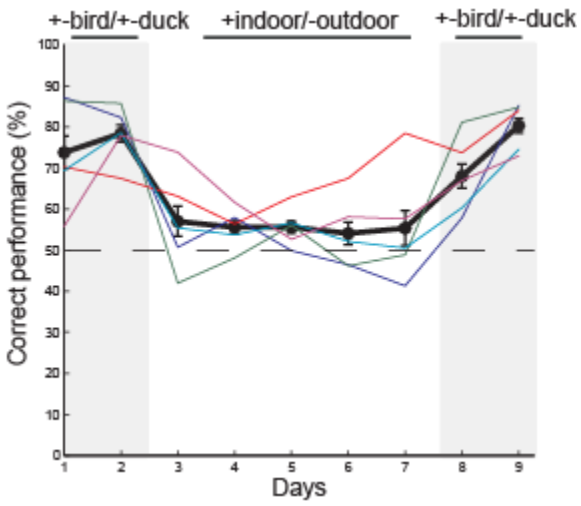


Fig 18 I Discrimination performance during conditioning to sound pairs bird/duck and indoor/outdoor.

The five mice with highest performance levels to bird/duck were selected and further conditioned to the sound pair indoor/outdoor. Finally, long-term memory formation was tested by introducing again the sound pair bird/duck. Colored lines represent mean correct performance of single mice and sessions, thick black line represents mean across mice and sessions, dotted black line represents chance level, grey areas represent conditioning to bird/duck, transparent area represents conditioning to indoor/outdoor, SEM, (n=5).

The previous tests had shown that only few mice acquired the task efficiently, thus, I decided to continue conditioning only with the five mice showing highest performance levels to the sound pair bird/duck. I tested again the influence of sound pair composition on discrimination performance and after 7 days of conditioning to bird/duck the third sound pair indoor/outdoor. As expected, performance levels dropped to chance. Surprisingly, this did not change over several days indicating that the new sound pair was not suitable for testing discrimination in mice. The reason for that is unknown. In previous experiments of my laboratory these sounds had been used successfully for classical fear conditioning, demonstrating that they can be at least perceived by mice. In order to test long-term memory formation of positive reinforced sounds I after four days of conditioning to indoor/outdoor again the sound pair bird/duck. I observed that already in the first session mouse performance increased and finally in the second session mice reached previous performance levels. The fact that this improvement of performance occurred at a much faster rate than after initial introduction could indicate memory formation of the sounds.

In summary, I find that it is possible to adapt go-no go tasks for auditory discrimination learning in mice. The general performance, however, appears to be lower than described for olfactory learning. It is possible that discrimination in different sensory modalities may be not be equally readily performed.

1.4 Discussion

1.4.1 Comparison of memory assessment using lick suppression and freezing behavior

The assessment of memory in behavioral experiments always utilizes a specific behavior as readout. However, different behavioral read outs could potentially influence the specificity and variability of the memory assessment. Therefore, I tested lick suppression and freezing in previously trained mice representing two alternative behavioral read outs in auditory fear conditioning.

In general, freezing is the absence of motion and represents an innate fear response. This leads to robust behavioral responses in fear conditioning memory tests. However, also more complex fear responses like the suppression of ongoing behavior can be used as behavioral read out. For example, lick suppression represents one of these instrumental behaviors, which requires little cognitive and motor skills (LeDoux 2000; Heffner, Koay et al. 2006). In general, in the natural environment of mice little licking can be observed. Therefore, most empirical studies use water deprivation of mice in order to enhance licking behavior. However, I decided to improve licking performance by using sweetened milk which is intrinsically attractive to mice (Neubert, King et al. 2008). In my experiments mice were familiarized to the drinking spout in the test chamber and habituated to sound presentation concomitant with liquid delivery which was initially distractive to mice. After habituation, I observed that visit durations during sound presentation were comparable to the levels observed in the no sound session and blank presentation, however, tended to be somewhat shorter. Furthermore, intrinsic sound properties influenced significantly licking behavior. For example, visits after presentation of gallop during habituation were similarly long as during no sound sessions. However, visits after presentation of keep were consistently shorter in all habituation sessions. This indicates that the sound keep seems to be intrinsically fearful and thus, may not be optimal for auditory fear conditioning in mice. Furthermore, this result clearly showed that mice were able to discriminate between complex sounds even without explicit training.

Conditioning induced strong suppression of licking during presentation of the paired stimulus gallop indicating successful conditioning. Licking in the no sound session or presentation of the blank after conditioning was only slightly reduced. Licking during presentation of sound stimuli which were not conditioned was either not affected (keep) or reduced to some degree (beet), indicating generalization. Interestingly, when the same mice were tested immediately afterwards using freezing as a behavioral read out, only to the target sound gallop elicited specifically fear responses. This indicates that the sensitivity of memory assessment can crucially depend on the behavioral assay used. In general, discrimination of complex sounds could be improved using a discriminative fear conditioning protocol (Herry, Ciocchi et al. 2008). Therefore, during the conditioning session the target sound is paired with a foot shock and immediately followed by presentation of a neutral sound in specific unpaired fashion. However, it remains to be determined if such a 'discriminative protocol' would improve discrimination of complex sounds since in the study of Herry et al. pure tones and white noise were used.

In summary, both behavioral read outs, freezing and lick suppression, lead to robust fear responses in mice after auditory fear conditioning. However, habituation for lick suppression tests lasts significantly longer than for freezing which includes commonly only 2-3 habituation sessions. Therefore, the latter learning paradigm is preferable for auditory fear conditioning. Furthermore, lick suppression was critically dependent on intrinsic sound properties as shown for the sound keep, in contrast to freezing which was a very robust.

1.4.2 Positive reinforcement of complex sounds using a modified second order learning paradigm

When investigating changes in the brain being correlated with learning behavior, it will become important to learn what specific aspects of changes reflect what specific aspects of the experience. For example, a putative change could be triggered by aspects of the CS or non-associative aspects of the US. In order to dissociate this, it would be desirable to reinforce the same neutral stimulus with either positive or negative reinforcers. As an approach to accelerate learning during positive reinforcement of complex sounds I chose a learning paradigm similar to classical second order conditioning. The advantage here is that the procedural aspects to the training could be temporally separated from the associative aspects. In classical second order conditioning a neutral stimulus (CS1) is paired with an unconditioned stimulus (US) in the first-order phase and in the second-order phase a second neutral stimulus (CS2) is paired with CS1. In general, auditory, visual or olfactory cues are used as neutral stimuli and the US is typically a foot shock which is similar as in Pavlovian fear condition. Furthermore, CS1 and CS2 are paired without US presentation and CS2 is tested without reinforcement.(Gewirtz and Davis 2000; Paschall and Davis 2002; Debiec, Doyere et al. 2006).

In my paradigm I reinforced both stimuli with reward which makes the learning paradigm different from classical order conditioning. However, this setup allowed me to measure learning while ongoing positive reinforcement. This is advantageous when mapping the learning curve in a novel paradigm. Furthermore, I used bright blue light flashes as CS1 since it may be similarly salient as a mild foot shock. Furthermore, it was shown in the study of Luskys et al. that mice could be efficiently trained to light as predictor for reward within few sessions (Luksys, Gerstner et al. 2009). In general, procedural aspects of the task are learned with the first stimulus light and finally the second stimulus sound is quickly introduced which allows to assess neural changes specifically due to auditory stimulus properties.

My tests showed that mice learned that light predicted reward within few sessions which is consistent with data from the study of Luskys et al. In general, after seven sessions mice reached constant correct performance levels between 80-90%. Afterwards, second order conditioning to a

complex sound was performed and within four sessions mice showed highly reliable responses to the sound indicating that they had learned the association between light and sound.

Next I considered that initial learning may already start in the first session before the full behavioral switch of latencies and tested second order conditioning to a complex sound in one 60 minute session. This would prolong the opportunity for mice to change their behavioral strategy within one session. On average, mice still entered the port with higher probabilities after light onset. However, in the last 30 minutes of the session some mice already started taking sound stimuli as predictor for reward. Nevertheless, the switch was still far from complete, as observed after multiple second order sessions.

One possible reason for the slow behavioral change could be that both stimuli were reinforced with reward which could reduce the pressure on changing behavioral strategies. A possible idea for the future following these lines could be to no longer offer reward during CS1 presentation. A potential concern here is that mice could stop licking at all for some period. Alternatively, one could still keep the reward contingency constant for CS1, however, offer a significantly higher amount of reward during CS2 presentation.

After establishing that initial changes in behavior can be achieved with second order approaches employing positive reinforcement even in a single session, it would be interesting to see in how far a classical second order conditioning learning paradigm would alter learning (Debiec, Doyere et al. 2006). Therefore, CS1 and CS2 would be paired in one session without reward and afterwards CS2 would be tested for its predictive value. A possible experiment could involve first conditioning to light and then one 15 min session pairing simultaneously CS2 and CS1 without reward delivery. In the test session CS2, two neutral sounds and one blank would be presented and probabilities of port entries after stimulus onset could be used as behavioral read-out. This would allow assessing second order memory formation and discrimination of complex sounds at the same time. Furthermore, random behavior could be directly inferred from visits after blank presentation. Additionally, the test session could be repeated 24 h hours later in order to assess long-term memory formation.

It is not clear which role the auditory cortex plays in the integration of information across multiple modalities. For example, in a classical second order conditioning paradigm the auditory

stimulus needs to be associated with the primary visual stimulus. Therefore, an associative network across different modalities needs to be established. It seems to be reasonable that the prefrontal cortex plays an important role in such computations (O'Reilly 2006). However, Ono et al. showed that lesions in the auditory cortex of rats impaired multimodal discrimination between sound and sound plus light (Ono, Kudoh et al. 2006). Therefore, it remains to be determined in how far the auditory cortex contributes to such a multimodal information processing. For example, it would be interesting to know if such associative networks would likely be stored in primary visual and auditory cortical areas or within one distinct associative area. A possible experiment to answer this question could involve second order conditioning in combination with auditory cortex lesions. Therefore, lesions at different time points before and after conditioning would give insight in necessity of the auditory cortex during establishment and storage of the multimodal information.

In summary, second order conditioning appears to be a promising venue to accelerate positive reinforcement paradigms. In particular it offers the opportunity to temporally separate procedural and associative aspects of the learning experience. It nevertheless will be necessary to optimize the detailed parameters of the paradigm to further improve learning performance in the single session.

1.4.3 Discrimination performance of mice in an operant go/no-go conditioning paradigm using complex sounds.

In order to link learning experiences with changes in the brain, it is obviously advantageous to employ fast learning paradigms that allow a sensitive comparison of neuronal function before and after learning. However, another important aspect of learning paradigms is the ability to assess the specificity of the memory. For example, learning paradigms that lead to conditioned behaviors only after presentation of the specific conditioned stimulus used during training (and not a broad range of more or less similar stimuli) allow parametric testing of the specificity of the memory. Examples of highly specific conditioning are shown in Figure 8. However, often simple fear conditioning paradigms involving less habituation sessions lead to relatively high generalization.

An explicit strategy to achieve specific behavioral responses to different stimuli is used in discriminative learning paradigms. Here, I adapted a discriminative go/no-go learning task that was implemented previously in the olfactory domain for learning of auditory stimuli (Abraham, Spors et al. 2004). This could be generally useful, since little is known about sensory processing and integration of complex sounds in mice. Studies investigating the discrimination of auditory stimuli (in different tasks than go/no-go) were mainly performed in rats using pure tones and frequency modulated pure tones (Heffner, Koay et al. 2006; Ono, Kudoh et al. 2006; Rybalko, Suta et al. 2006).

Mice could be trained in several sessions to performance levels of 80-90%. However, the fraction of mice achieving these high levels (~one third) was lower as compared to olfactory discrimination study of Abraham et al ((Abraham, Spors et al. 2004). What could be possible reasons for this difference? Two important parameters were different in their study: (1) Higher number of trials per session, possibly due to water deprivation. However, I observed that even prolonged training over many sessions, compensating for lower trial rates in a given session, did not improve the performance of mice. (2) Olfactory stimuli instead of auditory cues. Several studies have shown that rodents possess superb discriminative abilities for olfactory stimuli (Uchida and Mainen 2003; Rinberg, Koulakov et al. 2006). My findings are compatible with the notion that discrimination of stimuli in one modality as compared to another may pose a

differently challenging task for the mice. A possible indication of this is that certain pairs of sound stimuli, although salient and in the hearing range of mice, were not discriminated even after several training sessions. Importantly, this finding was obtained in mice which showed good discrimination performance for a different sound pair previously. Similarly, in a study of Ryabalko et al. water deprived rats were trained to discriminate between falling and rising frequency modulated tones and rats reached on average only performance levels of 60-70% (Rybalko, Suta et al. 2006). Also in the visual domain Andermann et al. analyzed visual discrimination in head fixed mice using an operant go/no-go task and calcium imaging of neural populations in the visual cortex (Andermann et al. 2010). The authors report that only half of the tested mice were able to acquire the task at all despite water deprivation mice and additional negative reinforcement of the negative sound. These findings stress the importance of the choice of stimuli to test discrimination behavior.

In the experimental design two groups of mice were run in parallel in which the reward contingency of reward / no reward was switched with respect to the pair of sounds. This allows detection of a possible bias induced by stimulus preferences. However, I did not observe any difference in performance between groups conditioned to beet/keep or bird/duck. Interestingly, mice which previously discriminated very well between bird/duck were not able to discriminate between indoor/outdoor, even after several days of testing. Thus, the composition of sound pairs significantly influenced discrimination performance Analysis of the frequency distributions of the various used sound pairs (indoor/outdoor, bird/duck, beet/keep; see appendix) revealed that the pair indoor/outdoor had the highest frequency overlap. This could point to a possible sound feature that may make discrimination for mice increasingly difficult, however, this clearly would need a more thorough investigation with a large number of stimulus pairs.

In summary, implementation of an auditory go/no-go discrimination task is possible in mice. This is interesting for future directions in which learning behavior could be combined with imaging approaches in head-fixed animals. However, the data suggest that the performance levels that had been described previously in olfactory discrimination tasks may not fully achieved in auditory discrimination. Implementation of additional negative reinforcers could possibly improve discrimination behavior.

1.5 **Outlook**

In this thesis several behavioral paradigms had been explored that will allow detailed analysis of specific aspects of auditory learning. In order to achieve a coherent understanding of sensory processing and memory formation, however, pure behavioral approaches will not be sufficient. It will be therefore important to combine defined learning behaviors with genetic and physiological approaches that will allow further opening of the black box ‘brain’. All of the various paradigms described in this thesis had been chosen to facilitate in one way or the other the combination with chronic imaging approaches that will hopefully be useful to pinpoint specific changes in the brain that are associated to various aspects of the learning paradigm.

2. A genetic strategy to identify synaptically coupled neurons

2.1 Introduction

2.1.1 Current trans-synaptic labeling approaches

The mammalian brain consists of billions of neurons which are highly variable in cell types and functions. Furthermore, this multitude of neurons forms a highly complex network. For example, it is estimated that a single neocortical neuron receives synaptic inputs from up to 10.000 other neurons. If we want to understand how the brain works we need to understand how neuronal activity propagates within these neuronal circuits. A major step towards a better understanding of the architecture of neuronal circuits would be the ability to map out all the input neurons to a given postsynaptic neuron.

In principle, the identification of interconnected neurons can be achieved by trans-neuronal tracers, which are substances that are transported across synaptic connections. Ideally such a neuronal tracer would need to fulfill specific criteria: First, it should be possible to introduce it in defined neuronal cell types, or single neurons. Second, the tracer should be transported only trans-synaptically in order to exclude labeling of nearby unconnected cells. Third, the transport should occur selectively in one direction via one synaptic step (monosynaptic) in order to map only direct connected neurons. In the past plant lectins, neurotropic viruses and bacterial toxins had been used for mapping major neuronal projection pathways in the brain (Nagy, Mar et al. 2009).

Wheat germ agglutinin (WGA)

In classical neuro-anatomical studies mostly wheat germ agglutinin (WGA), which naturally does not occur in the brain, has been used. WGA binds particular glycoproteins on neuronal surfaces, is transported trans-synaptically and is subsequently visualized with immunohisto-chemical methods. Classically, injections of the pure protein in defined brain regions had been used. However, this approach caused severe inflammations and could lead to unspecific labeling

of unrelated pathways. In recent years, WGA was endogenously expressed under neuron specific promoters in transgenic mice and flies or introduced to the model systems using adeno-viral vectors. However, transfer across synapses was always critically dependent on the expression level of the protein in first order neurons. Furthermore, the transport was bidirectional and involved multiple synapses (Yoshihara 2002).

Rabies virus

Another elegant viral strategy for labeling monosynaptic, direct neuronal connections was developed by Wickersham and colleagues in 2007 based on rabies viruses. The wild type virus spreads through the central nervous system specifically in trans-synaptic, retrograde manner. Wickersham et al modified the virus in two important aspects: First, they prevented the virus to spread beyond initially infected cells. For this the essential envelope glycoprotein G responsible for neuron specific binding is deleted from the virus genome and renders it replication deficient. In fact, this glycoprotein was replaced by a reporter gene which is then expressed instead in high levels in transfected cells.

Secondly, in order to direct viral uptake only to defined subsets of neurons a pseudotyped viral variant is produced. In this case the virus is grown in cells that express the envelope protein EnvA of the avian retrovirus ASLV-A that can functionally be incorporated in the virus capsid and complement the missing glycoprotein G. However, after injection the virions can only enter neurons which have been specifically engineered to express the cognate TVA-receptor on their cell surface.

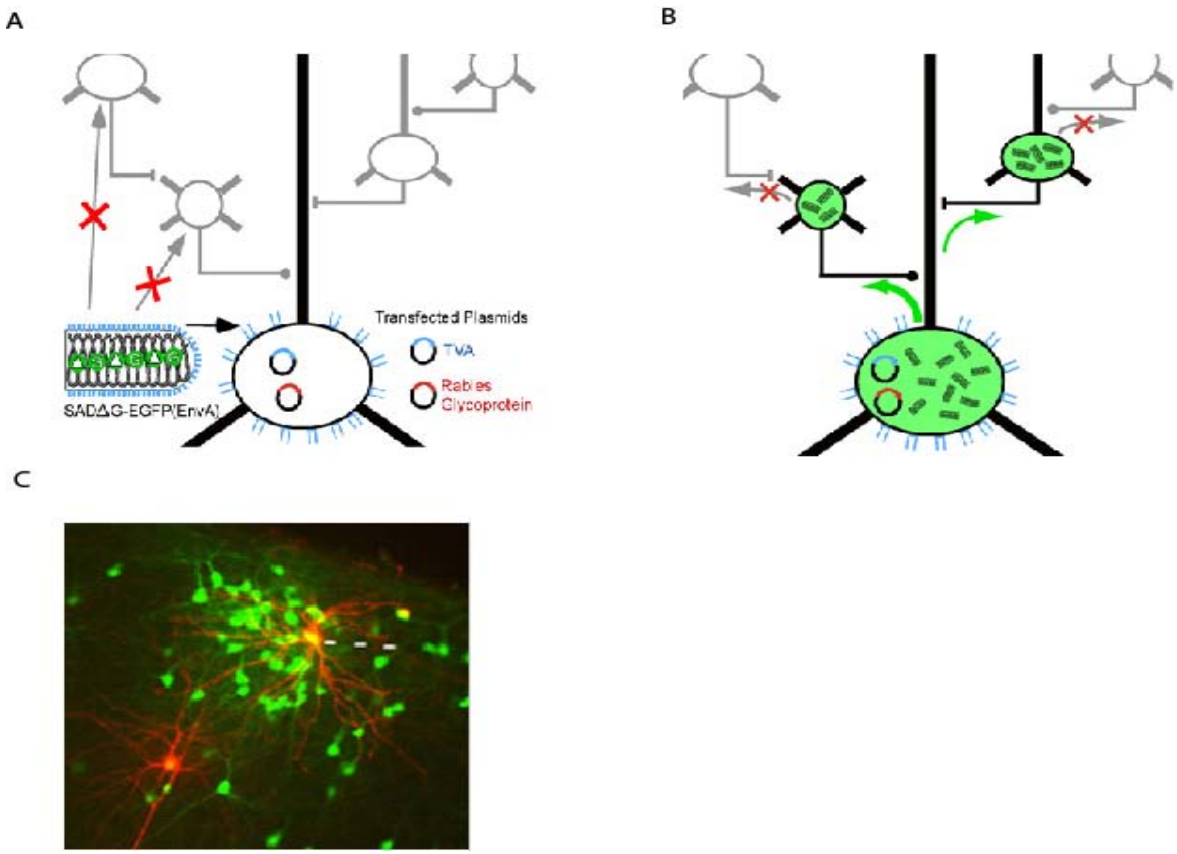


Figure 19 I Monosynaptic labeling of interconnected neurons using pseudotyped rabies virus.

A ASLV-A-pseudotyped rabies virus [SADDG-EGFP (EnvA)] infects mammalian neurons in which the gene for ASLV-A's receptor, TVA, has been introduced and expressed. Additional supply with an expression plasmid for the rabies virus glycoprotein enables spread to synaptically coupled cells. B Complementation with the rabies virus glycoprotein allows the virus to spread to directly presynaptic neurons. These cells all express the EGFP encoded in the viral genome, but the virus cannot spread beyond these cells C Long-range viral spread from single initially infected neurons (red), clusters of infected cells surrounding isolated putatively postsynaptic ones (green), figure adapted from Wickersham et al. 2007.

Currently this is the only technique which provides high expression levels of the transgenes, targeted infection of genetically specified neurons and specific monosynaptic transfer (Wickersham, Sullivan et al.). However, the system has still major drawbacks: So far the vector has been only tested *in vitro* neuronal brain slices. Furthermore, viral infection is not necessarily limited to one single cell. Most importantly, the targeted neuron will produce functional virions at high levels and this likely will affect the health of that neuron. Also, the system is rather complex furthermore limiting its applicability.

Tetanus toxin

The tetanus toxin belongs together with seven other botulinum toxins to the family of clostridial neurotoxins. It is produced by the bacterium Clostridium *tetani*. It germinates in tissue lesion and is the causative agent of tetanic spastic paralysis. The toxin enters nerve terminals at the neuromuscular junction, is selectively retrogradely transported to the spinal cord and blocks the neurotransmitter release in inhibitory neurons. This leads to disinhibition of motoneurons and spasm of the muscles. In the last few years this neurotoxin, although potentially harmful, came into focus of neuroscientific research. Its special ability to enter nerve terminals in retrograde fashion makes it an optimal tracer molecule for mapping neural circuits.

Uptake and transport of tetanus toxin

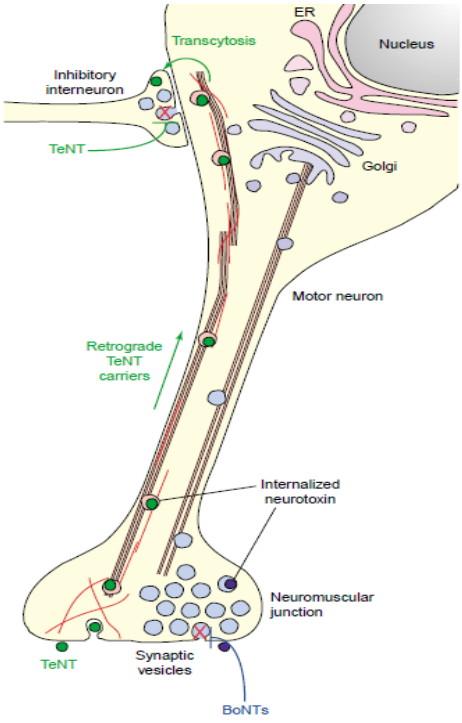


Figure 20 I Schematic view of a mammalian motoneuron and an interacting spinal inhibitory interneuron.

The sites of action of tetanus (TeNT; green) and botulinum neurotoxins (BoNTs; blue) are shown, together with their specific intracellular trafficking route, figure adapted from Lalli et. al 2003.

The tetanus toxin binds specifically to polysialogangliosides at the plasma membrane of the neuromuscular junction and is uptaken by misusing the synaptic vesicle recycling machinery. After axonal retrograde transport to the soma of the motoneuron in the spinal cord the toxin is exocytosed at the postsynaptic membrane of the motoneuron and uptaken at the presynaptic membrane of the inhibitory interneuron. Once released to the cytosol it cleaves specifically the synaptic vesicle associated membrane protein (VAMP) Synaptobrevin and blocks the neurotransmitter release. This causes spasm of the muscles.

Structure and function of tetanus toxin

The tetanus neurotoxin relates to the family of zinc endoproteases. Crystal structure analysis showed that it consists of two distinct domains, a 50 kD Light chain and a 100 kD heavy chain (Binz and Rummel 2009).

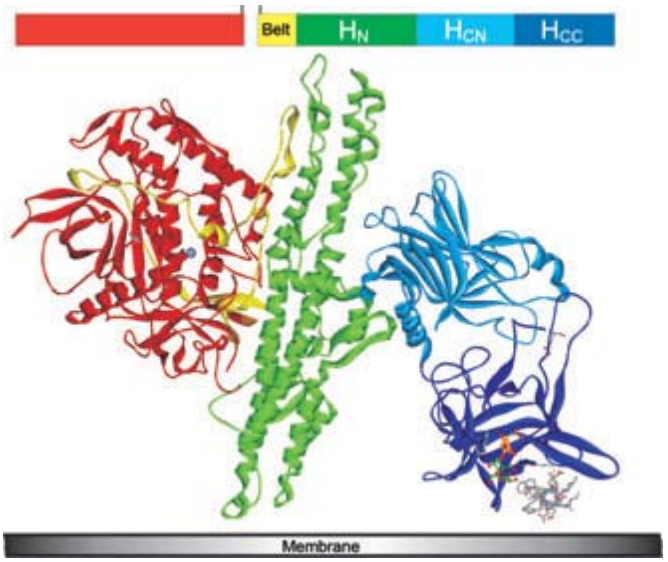


Figure 21 I Exemplary schematic and crystallographic presentation of the domain architecture of botulinum neurotoxin /B.

Light chain (red), heavy chain parts (blue, green), neuronal plasma membrane, figure adapted from Binz et al. 2009.

It was shown in several studies that neuron specific binding and retrograde transport of the tetanus toxin is due to the activity of the heavy chain (TTC) whereas the toxic action depends solely on the zinc protease activity of the light chain. This cleaves specifically the synaptic

vesicle associated protein (VAMP) Synaptobrevin and thereby blocks neurotransmitter release in inhibitory interneurons of the spinal cord. (Boquet and Duflot 1982; Matteoli, Verderio et al. 1996; Pellizzari, Rossetto et al. 1999; Lalli, Bohnert et al. 2003; Montecucco, Rossetto et al. 2004; Montecucco, Schiavo et al. 2005; Binz and Rummel 2009)

Dissociating the two domain structure of the protein leads also to separation of its toxic and non-toxic component. Coen et al. 1997 produced a hybrid protein between the heavy chain of tetanus toxin and β-galactosidase enzyme. It retained specific the neuronal uptake and retrograde transport *in vitro* and *in vivo* studies.(Coen, Osta et al. 1997)

Similarly, Maskos et al. 2002 expressed a fusion protein between green fluorescent protein (GFP) and tetanus toxin heavy chain in transgenic mice in defined subsets of neurons. Co-expression of an *nlacZ* reporter gene was used to discriminate between cells expressing the construct and the ones receiving it. They could show that trans-synaptic transport occurred specficially in retrograde direction. (Maskos, Kissa et al. 2002)

The Cre/*loxP* system

Trans-synaptic transport of a marker like GFP can facilitate the detection of connected neurons, however, this heavily relies on the fact that sufficient amounts of GFP would be trans-synaptically transported to allow reliable detection. Ideally, an amplification step would be included here that would increase the signal. This may be of particular importance when the connections of a single neuron should be mapped and likely the number of trans-synaptically transported molecules is low. Furthermore, it would be desirable to not only be able to label connected neurons, but to also potentially express a wide variety of genes of interest that would allow their manipulation.

The Cre/*loxP* recombinase system is one of the most powerful tools for genetic engineering nowadays since it allows conditional gene expression *in vivo*. The site-specific Cre recombinase has been isolated from bacteriophage P1 and naturally ensures stable maintenance of bacteriophage P1 plasmids in host bacteria. It recombines DNA at specific 35 bp DNA target sequences called *loxP* sites. A short crossing over region within the sequence determines its orientation leading to direct or inverted *loxP* repeats. Cre catalyzes the integration and excision

of DNA sequences between direct repeats and the inversion of DNA sequences between inverted repeats. (Kilby, Snaith et al. 1993)

For conditional gene expression *in vivo* a *loxP*-flanked STOP region downstream of a ubiquitously active promoter prevents the expression of any gene of interest following it. Upon Cre-excision the gene comes under the control of the promoter and starts to be expressed. (Nagy 2000). Cre-expression can be controlled using tissue or temporal specific promoters. Thus, the Cre/*loxP* system has been widely applied in different ways for conditional gene expression *in vivo* (Nagy, Mar et al. 2009).

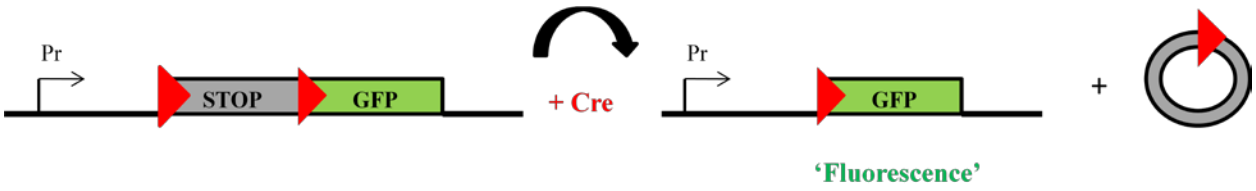


Figure 22 I Conditional gene expression using the Cre/*loxP* system.

Cre-recombinase excises the STOP region between two direct *loxP* sequences (red triangles). GFP comes under the control of the preceding promoter and is expressed leading to fluorescence.Pr promoter, GFP green fluorescent protein.

Indeed, there is recent evidence that Cre-recombinase can be transported trans-synaptically: This year, Gradinaru et al. published a fusion protein between WGA and Cre-recombinase for transcellular gene activation based on a two adeno-viral vector strategy (Gradinaru et al. 2010). They injected a WGA-Cre virus in one brain area and a second Cre-dependent virus in a supposedly interconnected one. They could show that Cre was transcellulary delivered with the help of WGA and activated gene expression only in connected neurons. Obviously this is a powerful approach for trans-synaptic gene activation *in vivo*. However, it is important to keep in mind that WGA can be transported antero- and retrogradely. Thus, having identified a connected neuron with this technique still does not give information if it is pre- or postsynaptically coupled. Additionally high levels of viral infection and expression are needed for long term *in vivo* applications which could have potential aversive effects (Gradinaru, Zhang et al.)

2.1.2 Central question

Despite considerable progress in labeling interconnected neurons, a system sensitive enough to selectively label input neurons of a single target neuron is still lacking. A potential solution that would combine the advantages of the sensitivity and versatility of Cre-recombinase and the selective retrograde transport of tetanus toxin for specific identification of input neurons could be a fusion protein of TTC and Cre-recombinase. Neurons expressing Cre-recombinase could be identified easily in a transgenic mouse line which allows conditional expression of a fluorescent protein depending on Cre mediated recombination (Madisen, Zwingman et al.) The strategy to assess the inputs of a single neuron would be targeted single-cell electroporation together with another expression plasmid coding for a fluorescent protein that would not be trans-synaptically transported. This would even allow functional characterization of the targeted neuron with cell-attached recordings before electroporation.

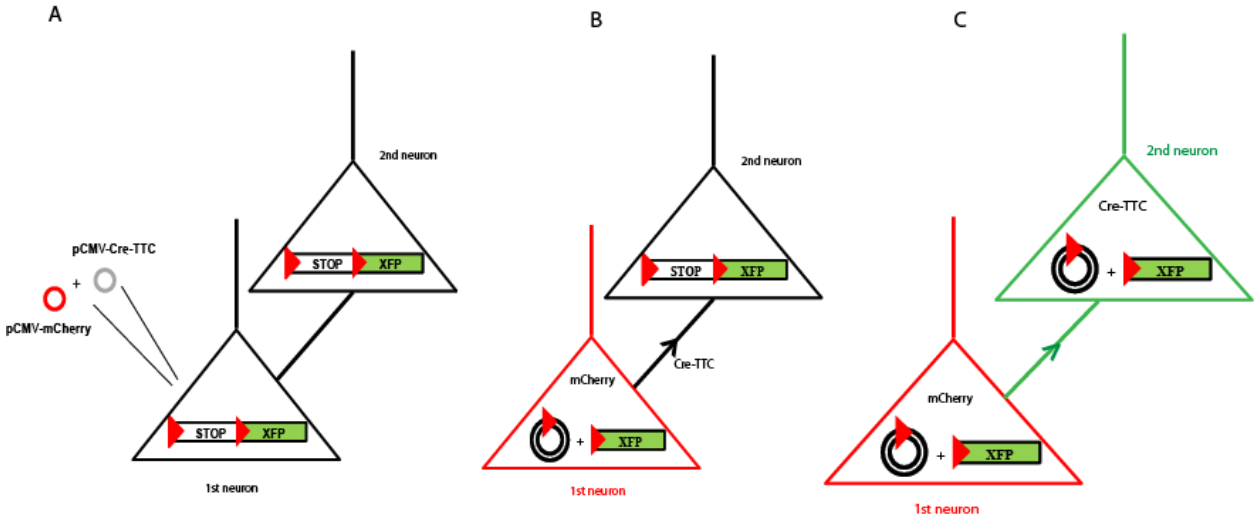


Figure 23 I Strategy to assess the inputs of a single neuron.

A Single-cell electroporation of CMV-Cre-TTC plasmid and CMV-mCherry control plasmid of the 1st neuron, B mCherry fluorescence and Cre recombination in the 1st neuron, retrograde transport of Cre-TTC to the 2nd interconnected neuron , C mCherry fluorescence in the first neuron, Cre recombination and XFP-fluorescence in the 2nd interconnected neuron.

The aim of this part of the thesis was to design and to construct expression vectors that would allow the expression of variants of a fusion protein of TTC and Cre-recombinase in order to test trans-synaptic transport and labeling efficacy.

The characterization of trans-synaptic transport is done in collaboration partners with expertise in single neuron electroporation.

2.2 Materials and Methods

2.2.1 Materials

Devices

C1000 Thermal Cycler, Bio-Rad Industries

Thermomixer compact, Eppendorf

Vortex Genie2, Scientific industries

Picofuge II, Stratagene

Centrifuge GR4i, Jouan

Centrifuge 5415 R, Eppendorf

Precast Gel system, Bio-Rad Industries

Epifluorescence microscope

General material

Gilson pipettes: P1, P20, P200, P1000

Gilson sterile pipette tips

Gilson pipetman

Eppendorf tubes (1.5ml)

Falcon tubes (10ml)

PCR tubes (50 ul)

tissue culture dishes

VWR Glassware

Semper care latex gloves

Enzymes

Fast Digest Restriction enzymes (Fermentas)

*EcoR*I, 5´…G^AATTC…3´

*Bam*HI, 5'...G^GATCC...3'

*Cla*I, 5'...AT^CGAT...3'

*Sal*I, 5'...G^TCGAC...3'

Xba I, 5'...T^CTAGA...3'

T4 DNA Ligase, 5U/µl (Fermentas)

Taq DNA Polymerase (native, with BSA; Fermentas)

Shrimp Alkaline Phosphatase (Fermentas)

Phusion® Hot Start High-Fidelity DNA Polymerase (Finnzymes)

Buffers/Solutions

10X FastDigest® Buffer, Fermentas

10X T4 DNA Ligase Buffer, Fermentas

10X *Taq* Buffer, Fermentas

5x Phusion HF Buffer, Finnzymes

dNTP Mix, 10 mM each, Fermentasα

6X DNA Loading Dye, Fermentas

GeneRuler™ 1 kb Plus DNA Ladder, 75-20,000 bp, Fermentas

GeneRuler™ 100 bp DNA Ladder, 100-1000 bp, Fermentas

10x PBS (2g/l KCl, 80g/l NaCl, 17.8g/l Na2HPO4, 2.4g/l KH2PO4, autoclaved)

20x SSC (175.3g/l NaCl 88.2g/l sodium citrate, autoclaved)

1xTE (10ml 1M Tris-Cl pH 7.5, 2ml 500mM EDTA pH 8.0 /l autoclaved)

Media

LB (Low Salt Luria Bertani)

10 g/l Tryptone, 5g/l Yeast Extract, 5 g/l NaCl, autoclaved;

Antibiotics where added after autoclaving to a final concentration of:

ampicilin 100µg/ml and kanamycin 50µg/ml;

Plates: LB media was prepared and 15g/l oxoid agar was added before autoclaving.

1% Agarose Gel

160 ml 1xTE buffer + 1,6g agarose

Kits

Wizard® SV Gel and PCR Clean-Up System (Promega)

QIAprep Spin Miniprep Kit

Zero Blunt® PCR Cloning Kit (Invitrogen)

Plasmid

CMV-MCS-plasmid (Stratagene)

Strains

E. coli DHα5 cell-line

HEK-293 cell-line

HEK- 293 reporter-cell-line with control plasmid containing *loxP*-sites and EGFP

2.2.2 Methods

Plasmid amplification and isolation

Cloning means using asexual reproduction to obtain organisms that are genetically identical. Bacteria can use plasmids to inherit e.g. resistance genes to different antibiotics to daughter cells. They are replicated independently of the chromosome whenever the cells divide. Under laboratory conditions E. *coli* duplicates approximately every 20 minutes. For cloning it is very efficient to use plasmids as vectors carrying a gene of interest. Whenever the bacterial cell replicates also the gene will be copied and a large number of identical copies will be produced.

For amplification of a plasmid, several individual colonies on an agar plate were picked with a toothstick. Each colony was subsequently transferred in a falcon tube containing 5 ml LB medium and incubated over night at 37°C. The medium contained an antibiotic for selection of bacteria that would carry the plasmid with the corresponding resistance gene.

After amplification of the plasmid in bacterial cell culture it needs to be extracted and purified. This means that the bacterial cell walls are broken up during lysis and the plasmid is separated from other cell components in solution like DNA, RNA proteins, lipids and carbohydrates. Plasmids were isolated with QIAprep Spin Miniprep Kit.

Digest and Ligation

Restriction endonucleases which cut the sugar-phosphate backbone of DNA at precise sites (restriction sites) can be used to obtain defined fragments of DNA. Bacteria use these enzymes as defense mechanism against foreign bacteriophages. This approach was used to excise defined DNA fragments for recombination or to test if the DNA sequence with the right base pair length was cloned into the vector. For such a control digest two restriction enzymes cutting at the 3’ and 5’ end of the target sequence were chosen to excise the DNA insert from the vector. Using gel electrophoresis they are separated according to their base pair length and are compared to a standard base pair ladder of known size. All fragments and vectors were digested with FastDigest® enzymes (Fermentas) or Restriction Enzymes (Roche) according to the supplied manual. Ligation describes the process of fusing fragments of DNA. Typically, a DNA fragment is fused in a vector backbone that allows subsequent transformation and amplification of the

resulting construct. After the digest and opening of a vector backbone, it was additionally dephosphorylated with shrimp alkaline phosphatase (SAP) for one hour at room temperature and then heated for 10 min at 75°C to inactivate the enzyme to prevent possible re-ligation of the vector without insertion of the desired fragment. Ligation was performed with T4 DNA Ligase, 5 U/µl (Fermentas) according to the supplied manual. The molar ratio of insert to vector of 1:1.5 was calculated with the equation.

$$\text{ng insert / ng vector} = \text{kb (insert) / kb (vector)}$$

E. *coli* transformation

The resulting recombinant DNA molecule is uptaken by bacterial cells during transformation. Generally this process is not very efficient because few cells incorporate a plasmid. To identify colonies actually containing a plasmid a resistance gene to a specific antibiotic like ampicilin or kanamycin is cloned into the vector. After transformation the cells are plated out onto agar plates containing the antibiotic. Only cells which received the plasmid will be able to grow and form colonies.

Transformation was performed using chemical competent E. *coli* DH5α-T1R cells. 200 µl cells were incubated with 7 µl ligation reaction for 30 min on ice. After a heat shock of 30 s at 42°C, 500 µl LB medium were added and cells were incubated 60 min at 37°C and 600rpm. Afterwards 100 µl of the reaction mixture was distributed on LB plates containing antibiotic depending on the resistance gene of the vector. Plates were incubated overnight at 37°C.

PCR

The polymerase chain (PCR) reaction can be used to obtain large quantities of specific DNA sequences. PCR requires a pair of primers that anneal to sites at either side of the required region of DNA. DNA polymerase synthesizes new DNA strands starting from each primer. Repeated cycles of denaturation of products, re-annealing of primers and extension give rise to an exponential amplification of DNA sequence between the two primers.

Thus, PCR can be also used to obtain the target gene from a plasmid. In this case, specific restriction sites are added at 3’ and 5’ end of the primers in order to enable the subsequent

ligation of target DNA and vector. Before that, both are first digested with restriction endonucleases. Then DNA ligases are used to join together target sequence and vector.

Standard PCR protocol of Finnzymes' Phusion® Hot Start High-Fidelity DNA Polymerase:

PCR reaction mix

Component	50 ul reaction	Final Concentration
H2O	add to 50 µl	
5x Phusion® HF Buffer*	10 µl	1x
10 mM dNTPs	1 µl	200 µM each
primer fw	x µl	1 µM
primer rv	x µl	1 µM
template DNA	x µl	
Phusion® Hot Start DNA Polymerase (2 U/µl)	0.5 µl	0.02 U/µl

Cycling conditions

Cycle step	Temperature	Time	Cycle number
Initial denaturation	98°C	30 s	
Denaturation Annealing Extension	98°C - 72°C	5 - 10 s - 15 - 30 s/kb	 x 34
Final extension	72°C 4°C	5 – 10 min hold	

Annealing temperature was optimized with respect to primer melting temperatures.

All Fragments were purified after gel electrophoresis with Wizard® SV Gel and PCR Clean-Up System (Promega).

PCR of iCre

iCre is a codon improved Cre recombinase The iCre-sequence was amplified by PCR of a plasmid with full length iCre sequence containing also a nuclear localization signal sequence.

PCR mix was produced according to Protocol for Finnzymes' Phusion® Hot Start High-Fidelity DNA Polymerase.

The following primers were used to produce iCre with and without NLS:

BamHI_NLS_iCre_fw

BamHI_iCre_fw

iCre_XbaI_rv

PCR of GFP, mCherry

The EGFP and mCherry-sequence was amplified by PCR of a plasmid containing full length EGFP or mCherry sequence.

PCR mix was produced according to Protocol for Finnzymes' Phusion® Hot Start High-Fidelity DNA Polymerase. The final concentration of template DNA was 50 ng/ µl.

The following primers were used:

GFP_mCherry_EcoRI_fw

GFP_mCherry_BamHI_rv

Overlap extension PCR of TTC

Tetanus Heavy Chain (TTC) gene was assembled by 2-step overlap extension PCR. Different overlapping oligonucleotides corresponding to the genomic TTC-sequence of C. *tetani* were ordered. First two small overlapping fragments T1 and T2 of≅ 250bp were produc ed using the following oligonucleotids:

T1: TTC fw1-fw5; TTC rv4-rv8;

T2: TTC fw6-fw9; TTC rv3-rv1;

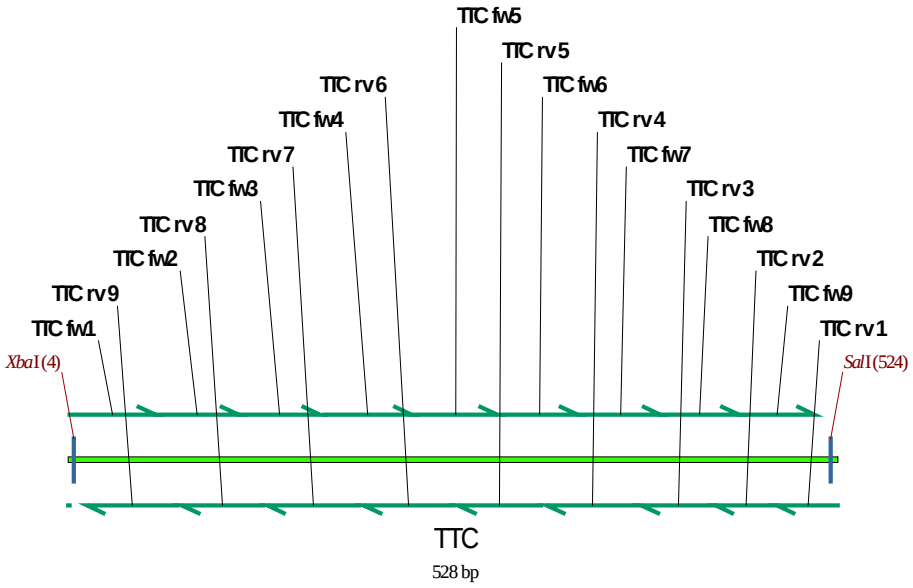


Figure 24 I Scheme of overlapping oligonucleotides corresponding to the genomic sequence of TTC.

In the second step the two overlapping fragments T1 and T2 were again amplified by PCR and the full length sequence of TTC (504bp) was obtained.

PCR reactions were produced according to Protocol for Finnzymes' Phusion® Hot Start High-Fidelity DNA Polymerase. They contained 15 pmol of the outer oligonucleotides which functioned as primer and 1.5 pmol of the inner oligonucleotides.

E. *coli* colony PCR

This was to test which colonies had uptaken the plasmid with the right insert.

PCR was performed with *Taq* DNA polymerase (Fermentas).

Primers were used corresponding to the 3' and 5' end of the target sequence

PCR reaction mix

Component	25 µl reaction	Final concentration
H2O	add to 25 µl	1x
Taq Buffer (10X)	0.2 µl	200 µM each
10 mM dNTP Mix	1 µl	1 µM
primer fw	x µl	1 µM
primer rv	x µl	
Taq Polymerase (5U/µl)	0.2 ul	0.005 U/µl

Cycling conditions

Cycle step	Temperature	Time	Cycle Number
Initial denaturation	94°C	5 min	
Denaturation Annealing Extension	94°C 58°C 68°C	30 s 20 1 min/kb	 x 34
Final extension	68°C 4°C	5 hold	

Different colonies were taken up with a toothpick from the LB-plate, added to the PCR reaction and mixed. Then PCR was performed according to the protocol of *Taq* polymerase.

Oligonucleotide assembly

Oligonucleotides corresponding to the full length sequence of the two signal peptide sequences GluR4 (Glutamate receptor subunit 4) and I cam (cell adhesion molecule class I) were ordered. They contained also the Kozak sequence ACC preceding the start codon to promote high-level translation in mammalian cells.

For oligonucleotide assembly 5 µl of each oligonucleotide stock solution (100µM) were mixed with 6 µl 20xSSC and filled up with water to a final volume of 25 µl. The reaction mix was heated for 5 min at 99°C and afterwards cooled down slowly to room temperature in the PCR cycler.

The fragments were purified using standard alcohol DNA precipitation protocol.

Cloning strategy

A pCMV-MCS plasmid (Stratagene) was used for cloning. It contains a multiple cloning site (MCS) which allows the insertion of different DNA fragments in specific order. The 0.4-kb cytomegalovirus (CMV) promoter upstream of the MCS is responsible for high level expression in mammalian cells. The ampicilin resistance gene is needed for selection of transformed bacterial colonies.

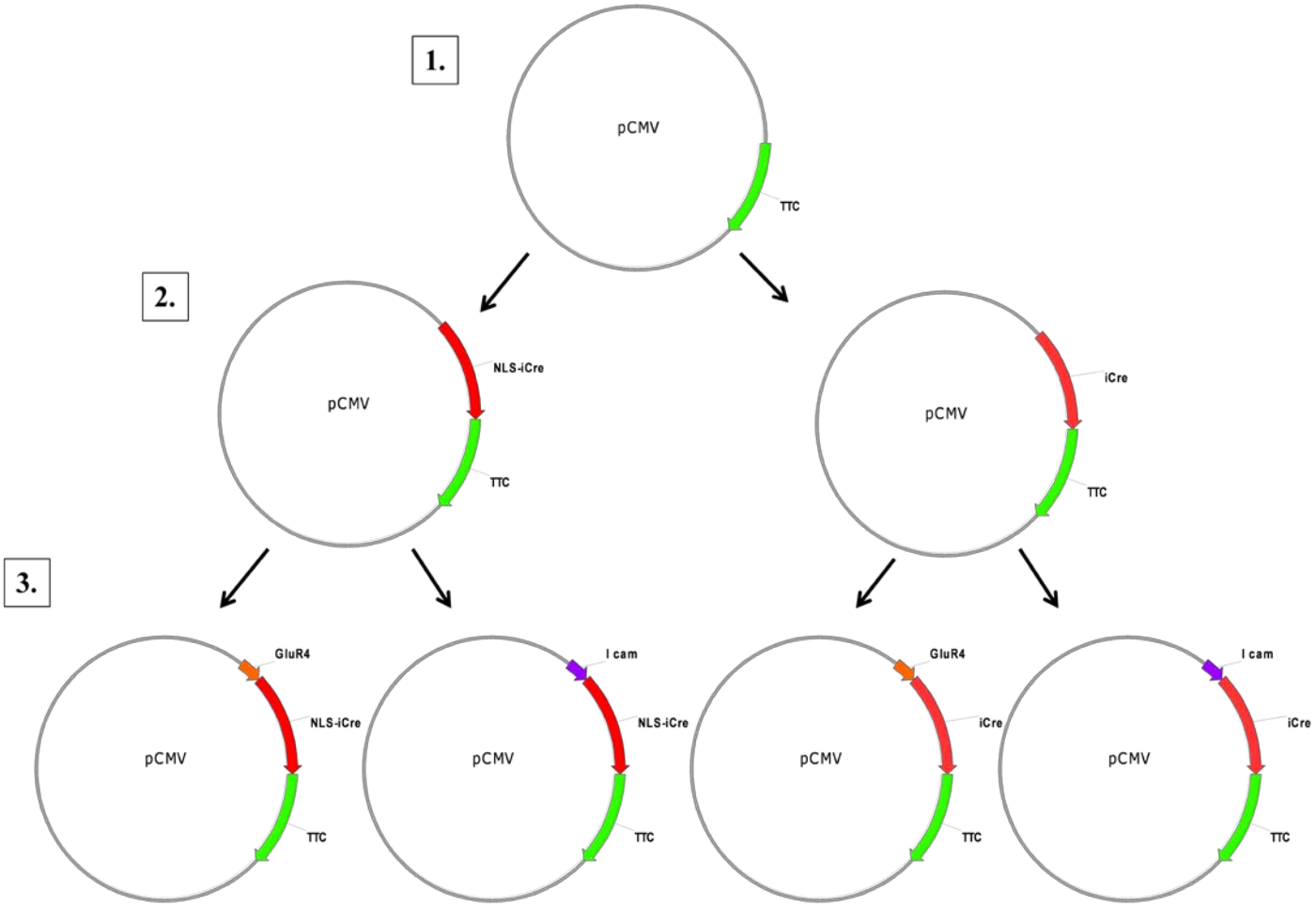


Figure 25 I Cloning strategy for plasmids containing fusion protein sequence of iCre-TTC.

First the TTC sequence was cloned into MCS of the CMV plasmid. In a second step the iCre sequence with and without NLS was introduced upstream of TTC resulting in two variants of the plasmid. In the third step signal peptide sequences I cam or GluR4 were cloned into constructs leading to four variable plasmids.

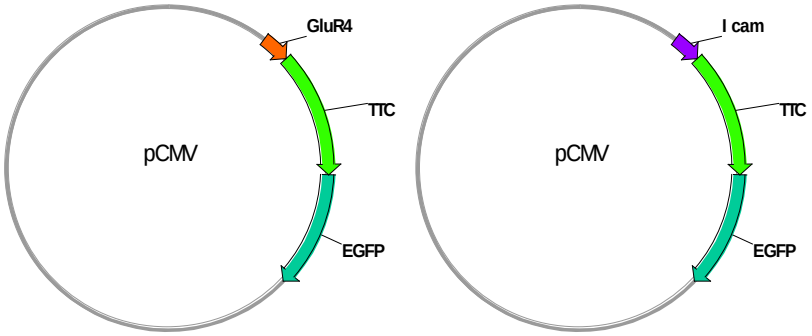


Figure 26 I Cloning strategy for plasmids containing the fusion protein sequence of EGFP-TTC.

The EGFP-TTC fusion protein (Maskos, Kissa et al. 2002) was produced first by excising the iCre sequence in the pCMV-GluR4- and pCMV-I cam–iCre-TTC plasmids. Then the EGFP sequence was cloned upstream of TTC into MCS the CMV plasmid.

Transfection of HEK 293-cells

The expression of a foreign gene is often performed in eukaryotic host cells since bacterial systems show different post-translational modifications. These are needed for the conversion of the primary product of translation into the mature, folded, biologically active protein. For expression in mammalian cells, like Human Embryonic Kidney 293 cells, a variety of expression vectors can be used. Most of them contain the promoter from the human cytomegalovirus (CMV) to drive high level, constitutive expression.

For expression analysis of plasmids containing iCre-TTC-sequences a HEK293-reporter-cell-line carrying a reporter plasmid with GFP and preceding loxP-flanked stop region was transfected. As positive control a plasmid containing functional iCre was transfected.

For expression analysis of plasmids containing EGFP-TTC sequences, EGFP or mCherry a HEK293-cell-line was transfected. As positive control a plasmid coding for functional EGFP was transfected. Untransfected cells served as negative control.

HEK293- cells were grown in cell culture dishes containing 5 ml DMEM growth medium 48 h at 37°C in the incubator. After the cells reached confluency of approximately 60% a calcium phosphate-based transfection protocol was performed. 5 µg of each plasmid were pipetted to 300 µl $CaCl_2$ in a 1.5 ml eppendorf tube. Then 300 µl 2xHBS were added to the DNA/ $CaCl_2$ mixture and incubated for 20 min at room temperature. Afterwards the DNA/ $CaCl_2$/HBS suspension was applied to the cell culture dishes in a dropwise fashion. After 5 hours incubation at 37°C DMEM medium was changed and cells were incubated 24 h for GFP/mCherry expression and 48 h for Cre expression.

Epifluorescence microscopy

The expression level of the fusion proteins was tested with an upright epifluorescence microscope, equipped with a 10x objective (NA, Zeiss Axioplan2 imaging), an HBO 100 mercury arc lamp (ZEISS) and a Coolsnap CCD camera (Photometrics). For imaging of EGFP fluorescence we used a Zeiss Excitation 460-490/Emission 500-550 fiterset and for mCherry a Zeiss Excitaion 540-580/Emission 590-650 fiterset. Images were acquired using Metamorph (Molecular Devices) image analysis software and stored to the hard drive of a computer.

2.3 **Results**

The following plasmids were produced:

1 pCMV-GluR4-NLS-iCre-TTC, 2 pCMV-GluR4-iCre-TTC, 3 pCMV-I cam-NLS-iCre-TTC,

4 pCMV-I cam-iCre-TTC, 5 pCMV-GluR4-EGFP-TTC, 6 pCMV-I cam-EGFP-TTC,

7 pCMV-EGFP, 8 pCMV-mCherry

2.3.1 pCMV-GluR4-NLS-iCre-TTC

This plasmid can be used to test trans-synaptic transport of iCre. It is similar to one described in Maskos et al. 2002 except that GFP was exchanged for iCre (with NLS). This construct should be tested in neurons requiring Cre-recombination for expression of a reporter gene. If this construct works, the reporter should be active in the electroporated neuron as well as in synaptically coupled neurons.

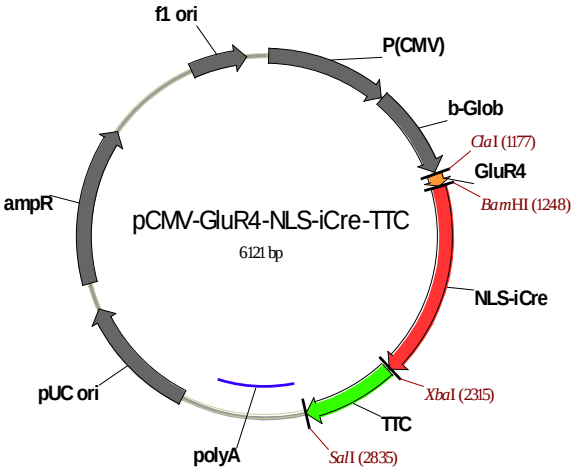


Figure 27 I Vector map CMV-GluR4-NLS-iCre-TTC plasmid.

pUC ori: *E. coli* origin of replication; ampR: ampicilin resitance gene; ori: f1 phage origin of replication allows the rescue of antisense ssDNA by a helper phage; P(CMV): human cytomegalovirus immediate early promoter for mammalian expression; b-Glob: β-globin gene; GluR4: glutamate receptor subunit 4, signal peptide sequence; NLS: nuclear localization signal; iCre: codon improved Cre-recombinase; TTC: tetanus heavy chain; polyA: polyadenylation signal for mammalian expression.

The TTC fragment was cloned into multiple cloning site (MCS) downstream of a 0.4-kb cytomegalovirus (CMV) promoter of the CMV-MCS plasmid (Stratagene). iCre was fused N-terminally to TTC. The GluR4 signal peptide sequence contained the Kozak sequence ACC preceding the start codon to promote high-level translation in mammalian cells. GluR4 was cloned into MCS upstream of iCre-TTC-sequence to produce the construct with the full-length DNA of the fusion protein GluR4-NLS-iCre-TTC.

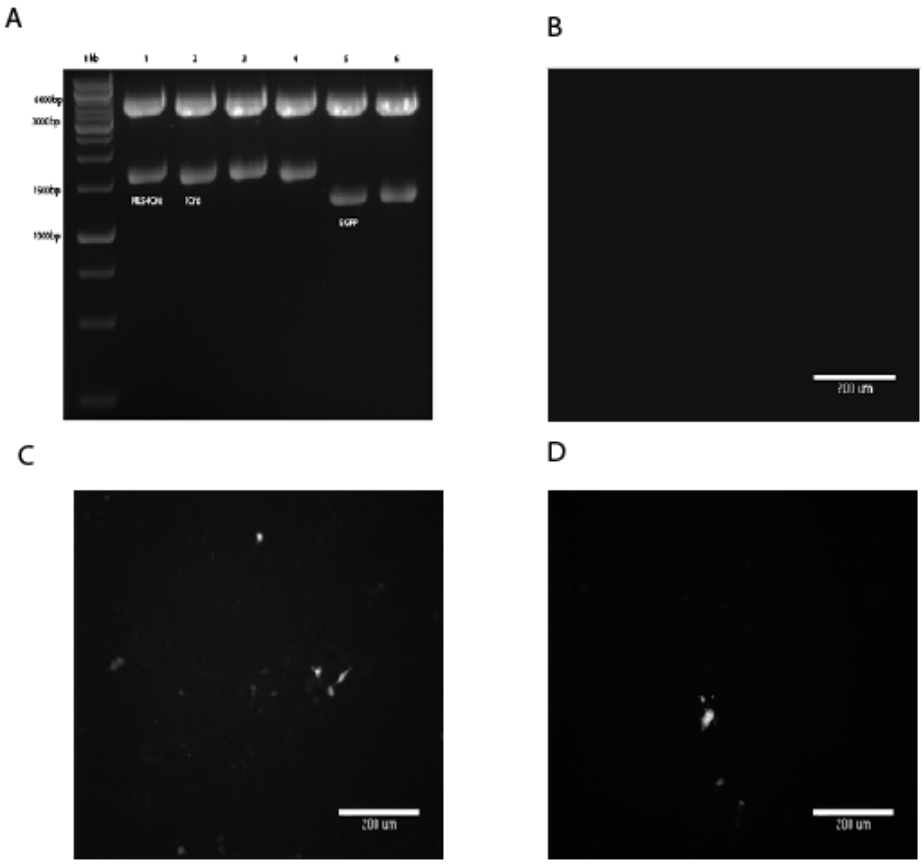


Figure 28 I Verification of CMV-GluR4-NLS-iCre-TTC plasmid.

A Control digest, line 1 represents DNA bands corresponding to vector and insert fragment size, B Transfection of HEK293-reporter-cell-line and epifluorescence microscope images, negative control, C as described in B, positive control with functional Cre-plasmid, C as described in B, test of CMV-GluR4-NLS-iCre-TTC plasmid.

The final construct was verified by sequencing the insert and a control digest using restriction endonucleases *Sal*I and *Cla*I with predicted fragments of 1653bp and 4468bp. To test the expression of the fusion protein a HEK293-reporter-cell-line carrying a reporter plasmid with GFP and preceding *loxP*-flanked stop region was transfected with the pCMV-GluR4-NLS-iCre-TTC plasmid. Cells were incubated for 48h to allow iCre-expression. As positive control cells were transfected with a control plasmid containing functional iCre. As negative control served untransfected cells. The expression level was tested by epifluorescence microscopy.

2.3.2. pCMV-GluR4-iCre-TTC

This plasmid can be used to test trans-synaptic transport of iCre. It is similar to one described in Maskos et al. 2002 except that GFP was exchanged for iCre. The lack of NLS may improve trans-synaptic transport. This construct should be tested in neurons requiring Cre-recombination for expression of a reporter gene. If this construct works, the reporter should be active in the electroporated neuron as well as in synaptically coupled neurons.

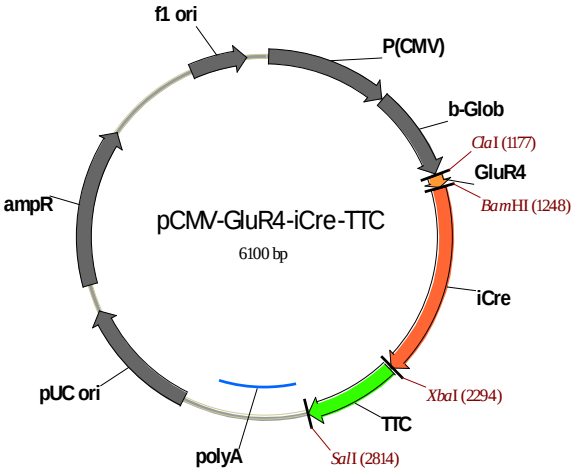


Figure 29 I Vector map of CMV-GluR4- iCre-TTC plasmid.

pUC ori: E. *coli* origin of replication; ampR: ampicilin resitance gene; ori: f1 phage origin of replication allows the rescue of antisense ssDNA by a helper phage; P(CMV): human cytomegalovirus immediate early promoter for mammalian expression; b-Glob: β-globin gene; GluR4: glutamate receptor subunit 4, signal peptide sequence; iCre: codon improved cre-recombinase; TTC: tetanus heavy chain; polyA: polyadenylation signal for mammalian expression.

The TTC fragment was cloned into multiple cloning site (MCS) downstream of a 0.4-kb cytomegalovirus (CMV) promoter of the CMV-MCS plasmid (Stratagene). iCre was fused N-terminally to TTC. GluR4 signal peptide sequence contained the Kozak sequence ACC preceding the start codon to promote high-level translation in mammalian cells. It was cloned into MCS upstream of iCre-TTC-sequence to produce the construct with the full-length DNA of the fusion protein GluR4- iCre-TTC.

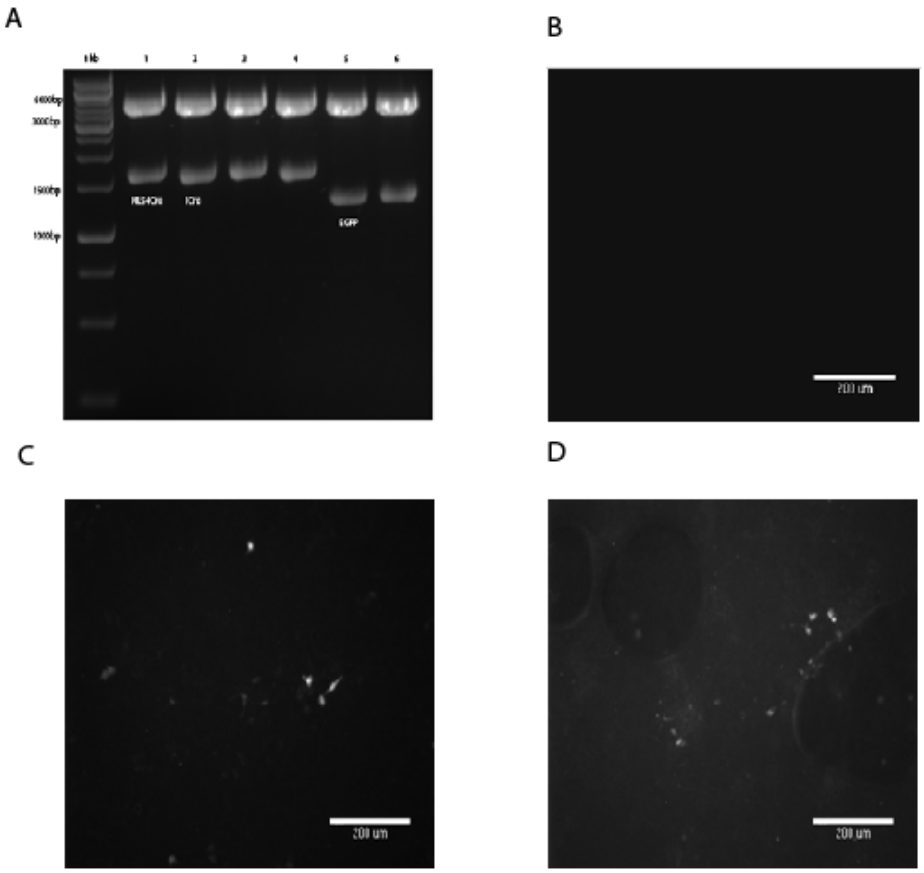


Figure 30 I Verification of CMV-GluR4-iCre-TTC plasmid.

A Control digest, line 2 represents DNA bands corresponding to vector and insert size, B Transfection of HEK293-reporter-cell-line and epifluorescence microscope images, negative control, C as described in B, positive control with functional Cre-plasmid, C as described in B, test of CMV-GluR4- iCre-TTC plasmid.

The final construct was verified by sequencing the insert and a control digest using restriction endonucleases *Sal*I and *Cla*I with predicted fragments of 1632bp and 4468bp. To test the expression of the fusion protein a HEK293-reporter-cell-line carrying a reporter plasmid with GFP and preceding *loxP*-flanked stop region was transfected with the pCMV-GluR4 -iCre-TTC plasmid. Cells were incubated for 48h to allow iCre-expression. As positive control cells were transfected with a control plasmid containing functional iCre. As negative control served untransfected cells. The expression level was tested by epifluorescence microscopy.

2.3.2 pCMV-I cam-NLS-iCre-TTC

This plasmid can be used to test trans-synaptic transport of iCre. This construct is similar to one described in Maskos et al. 2002, except that GFP was exchanged for iCre. This construct in comparison with (1) allows testing if the I cam signal sequence targeting the fusion protein to the exocytic compartment is better suited than the GluR4 signal sequence. This construct should be tested in neurons requiring Cre-recombination for expression of a reporter gene. If this construct works, the reporter should be active in the electroporated neuron as well as in synaptically coupled neurons.

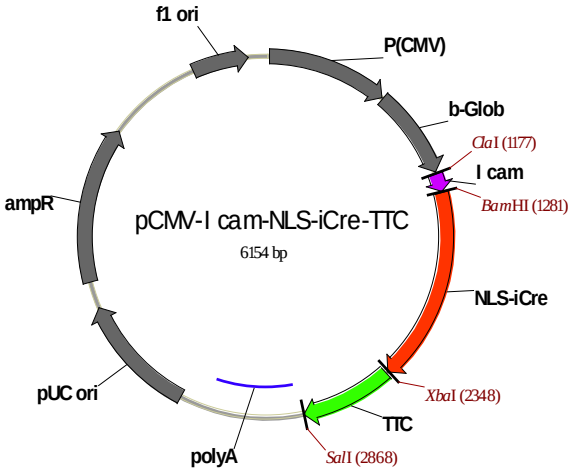


Figure 31 I Vector map of CMV-I cam-NLS-iCre-TTC plasmid.

pUC ori: E. *coli* origin of replication; ampR: ampicilin resitance gene; ori: f1 phage origin of replication allows the rescue of antisense ssDNA by a helper phage; P(CMV): human cytomegalovirus immediate early promoter for mammalian expression; b-Glob: β-globin gene; I cam: cell adhesion molecule class I, signal peptide sequence; NLS: nuclear localization signal; iCre: codon improved Cre-recombinase; TTC: tetanus heavy chain; polyA: polyadenylation signal for mammalian expression.

The TTC fragment (504bp) was cloned into multiple cloning site (MCS) downstream of a 0.4-kb cytomegalovirus (CMV) promoter of the CMV-MCS plasmid (Stratagene). iCre was fused N-terminally to TTC and is under control of a ubiquitous CMV promoter. The I cam signal peptide sequence contained the Kozak sequence ACC preceding the start codon to promote high-level translation in mammalian cells. Finally, I cam was cloned into MCS upstream of NLS-iCre-TTC-sequence to produce the construct with the full-length DNA of the fusion protein I cam-NLS-iCre-TTC.

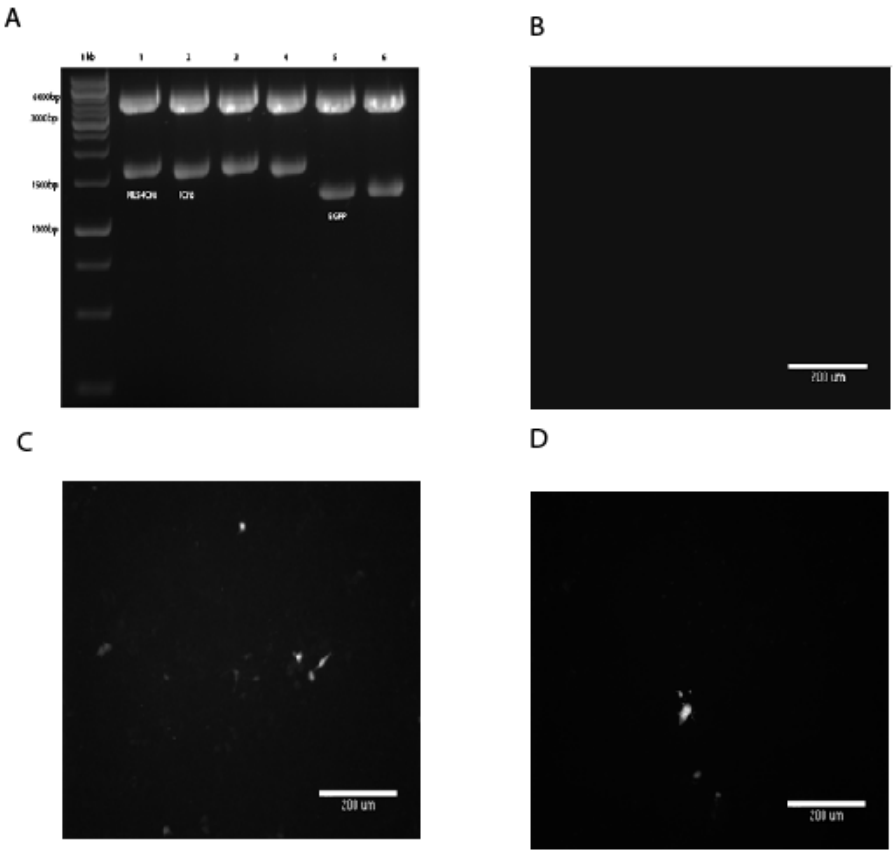


Figure 32 I Verification of CMV-I cam-NLS-iCre-TTC plasmid.

A Control digest, line 3 represents DNA bands corresponding to vector and insert fragment size, B Transfection of HEK293-reporter-cell-line and epifluorescence microscope images, negative control, C as described in B, positive control with functional Cre-plasmid, C as described in B, test of CMV-I cam-NLS-iCre-TTC plasmid.

The final construct was verified by sequencing the insert and a control digest using restriction endonucleases *Sal*I and *Cla*I with predicted fragments of 1686bp and 4468bp. To test the expression of the fusion protein a HEK293-reporter-cell-line carrying a reporter plasmid with GFP and preceding *loxP*-flanked stop region was transfected with the I cam-NLS-iCre-TTC-CMV plasmid. Cells were incubated for 48h to allow proper iCre-expression. As positive control cells were transfected with a control plasmid containing functional iCre. As negative control served untransfected cells. The expression level was tested by epifluorescence microscopy.

2.3.3 pCMV-I cam-iCre-TTC

This plasmid can be used to test trans-synaptic transport of iCre. This construct is similar to one described in Maskos et al. 2002 PNAS, except that GFP was exchanged for iCre. This construct in comparison with (2) allows testing if the I cam signal sequence targeting the fusion protein to the exocytic compartment is better suited than the GluR4 signal sequence. The lack of NLS may improve trans-synaptic transport. This construct should be tested in neurons requiring Cre-recombination for expression of a reporter gene. If this construct works, the reporter should be active in the electroporated neuron as well as in synaptically coupled neurons.

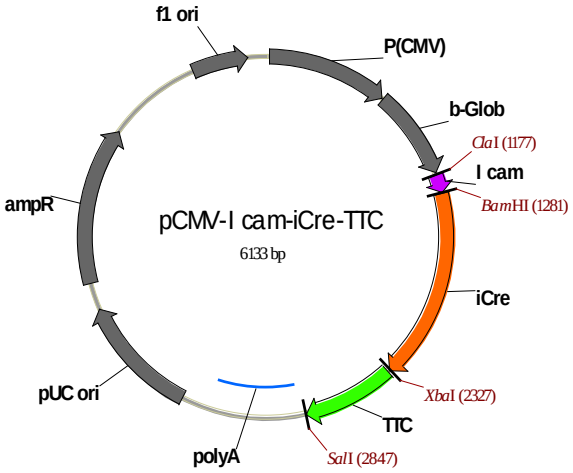


Figure 33 I Vector map CMV-I cam-iCre-TTC plasmid.

pUC ori: E. *coli* origin of replication; ampR: ampicilin resitance gene; ori: f1 phage origin of replication allows the rescue of antisense ssDNA by a helper phage; P(CMV): human cytomegalovirus immediate early promoter for mammalian expression; b-Glob: β-globin gene; I cam: cell adhesion molecule class I, signal peptide sequence; iCre: codon improved Cre-recombinase; TTC: tetanus heavy chain; polyA: polyadenylation signal for mammalian expression.

The TTC fragment(504bp) was cloned into multiple cloning site (MCS) downstream of a 0.4-kb cytomegalovirus (CMV) promoter of the pCMV-MCS plasmid (Stratagene). iCre was fused N-terminally to TTC and is under control of a ubiquitous CMV promoter. The I cam signal peptide sequence contained the Kozak sequence ACC preceding the start codon to promote high-level translation in mammalian cells. Finally, I cam was cloned into MCS upstream of iCre-TTC-sequence to produce the construct with the full-length DNA of the fusion protein Icam- iCre-TTC.

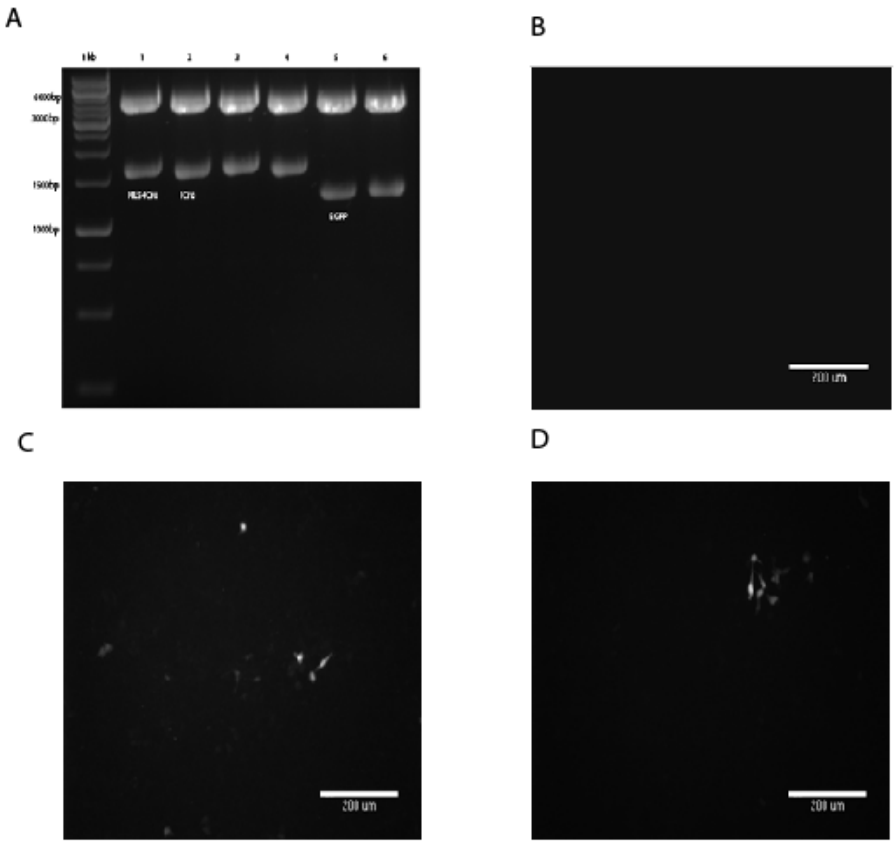


Figure 34 I Verification of CMV-I cam-iCre-TTC plasmid.

A Control digest, line 4 represents vector and insert DNA fragment bands, B Transfection of HEK293-reporter-cell-line and epifluorescence microscope images, negative control, C as described in B, positive control with functional Cre-plasmid, C as described in B, test of CMV-I cam-iCre-TTC plamid.

The final construct was verified by sequencing the insert and a control digest using restrictionendonucleases *Sal*I and *Cla*I with predicted fragments of 1665bp and 4468bp. To test the expression of the fusion protein a HEK293-reporter-cell-line carrying a reporter plasmid with GFP and preceding *loxP*-flanked stop region was transfected with the pCMV-I cam -iCre-TTC plasmid. Cells were incubated for 48h to allow iCre-expression. As positive control cells were transfected with a control plasmid containing functional iCre. As negative control served untransfected cells. The expression level was tested by epifluorescence microscopy.

2.3.4 pCMV-GluR4-EGFP-TTC

This plasmid can be used to test trans-synaptic transport of EGFP (with GluR4 signal sequence). This fusion protein is identical to one described in Maskos et al 2002. If this construct works, EGFP fluorescence should be visible in the electroporated neuron and in synaptically coupled neurons.

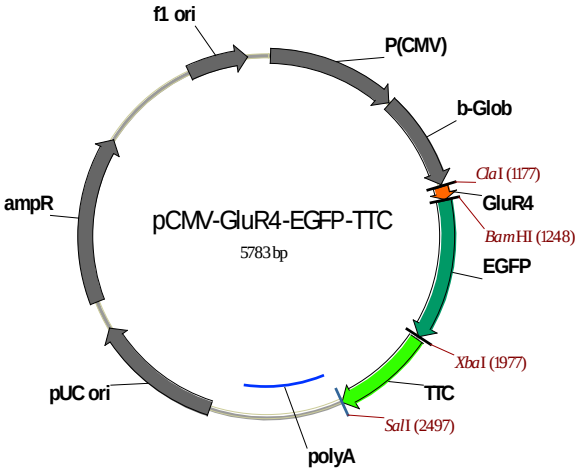


Figure 35 I Vector map of CMV-GluR4-EGFP-TTC plasmid.

pUC ori: E. *coli* origin of replication; ampR: ampicilin resitance gene; ori: f1 phage origin of replication allows the rescue of antisense ssDNA by a helper phage; P(CMV): human cytomegalovirus immediate early promoter for mammalian expression; b-Glob: β-globin gene; GluR4: glutamate receptor subunit 4, signal peptide sequence; EGFP: enhanced green fluorescent protein; TTC: tetanus heavy chain; polyA: polyadenylation signal for mammalian expression.

EGFP was fused N-terminally to TTC and is under control of a ubiquitous CMV promoter. The GluR4 signal peptide sequence contained the Kozak sequence ACC preceding the start codon to promote high-level translation in mammalian cells. Finally, GluR4 was cloned into MCS upstream of EGFP-TTC-sequence to produce the construct with the full-length DNA of the fusion protein GluR4-EGFP-TTC.

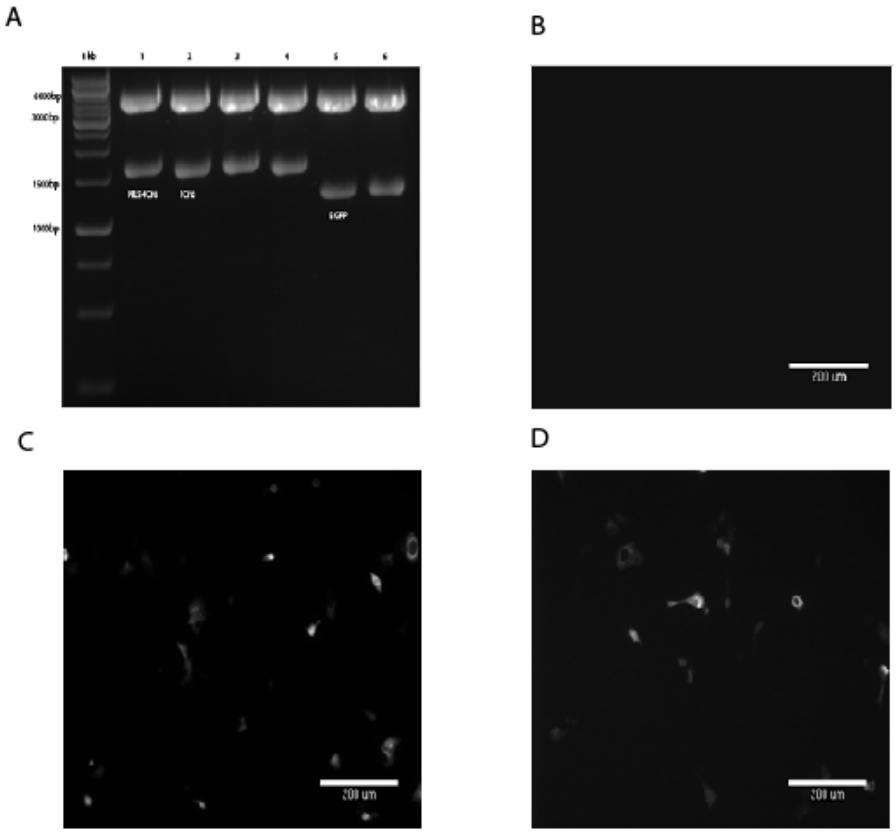


Figure 36 I Verification of CMV-GluR4-EGFP-TTC plasmid.

A Control digest, line 5 represents DNA bands corresponding to vector and insert fragment size, B Transfection of HEK293-cell-line and epifluorescence microscopy images, negative control, C as described in B, positive control with functional EGFP-plasmid, C as described in B, test of CMV-GluR4-EGFP-TTC plasmid.

The final construct was verified by sequencing the insert and a control digest using restriction endonucleases *Sal*I and *Cla*I with predicted fragments of 1315bp and 4468bp. To test the expression of the fusion protein a HEK293-cell-line was transfected with the pCMV-GluR4-EGFP-TTC plasmid. Cells were incubated for 24h to allow proper EGFP-expression. As positive control cells were transfected with a control plasmid containing functional EGFP. As negative control served untransfected cells. The expression level was tested by epifluorescence microscopy.

2.3.5 pCMV- I cam-EGFP-TTC

This plasmid can be used to test transsynaptic transport of EGFP (with I cam signal sequence). This construct in comparison with (5) allows testing if the I cam signal sequence targeting the fusion protein to the exocytic compartment is better suited than the GluR4 signal sequence. This fusion protein is identical to one described in Maskos et al 2002. If this construct works, EGFP fluorescence should be visible in the electroporated neuron and in synaptically coupled neurons.

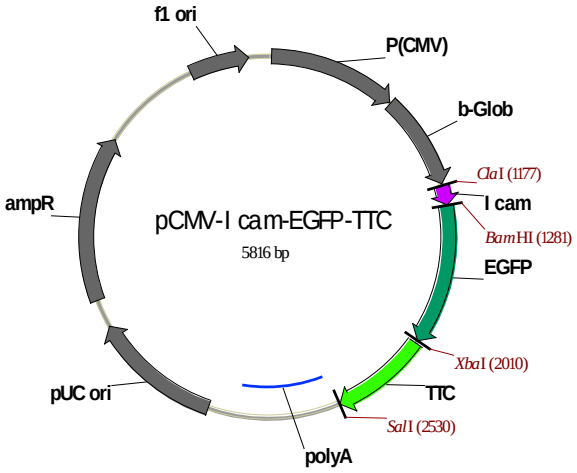


Figure 37 I Vector map of CMV-I cam- EGFP-TTC plasmid.

pUC ori: E. *coli* origin of replication; ampR: ampicilin resitance gene; ori: f1 phage origin of replication allows the rescue of antisense ssDNA by a helper phage; P(CMV): human cytomegalovirus immediate early promoter for mammalian expression; b-Glob: β-globin gene; I cam: cell adhesion molecule class I, signal peptide sequence; EGFP: enhanced green fluorescent protein; TTC: tetanus heavy chain; polyA: polyadenylation signal for mammalian expression.

EGFP was fused N-terminally to TTC and is under control of a ubiquitous CMV promoter. The I cam signal peptide sequence contained the Kozak sequence ACC preceding the start codon to promote high-level translation in mammalian cells. Finally, I cam was cloned into MCS upstream of EGFP-TTC-sequence to produce the construct with the full-length DNA of the fusion protein I cam-EGFP-TTC.

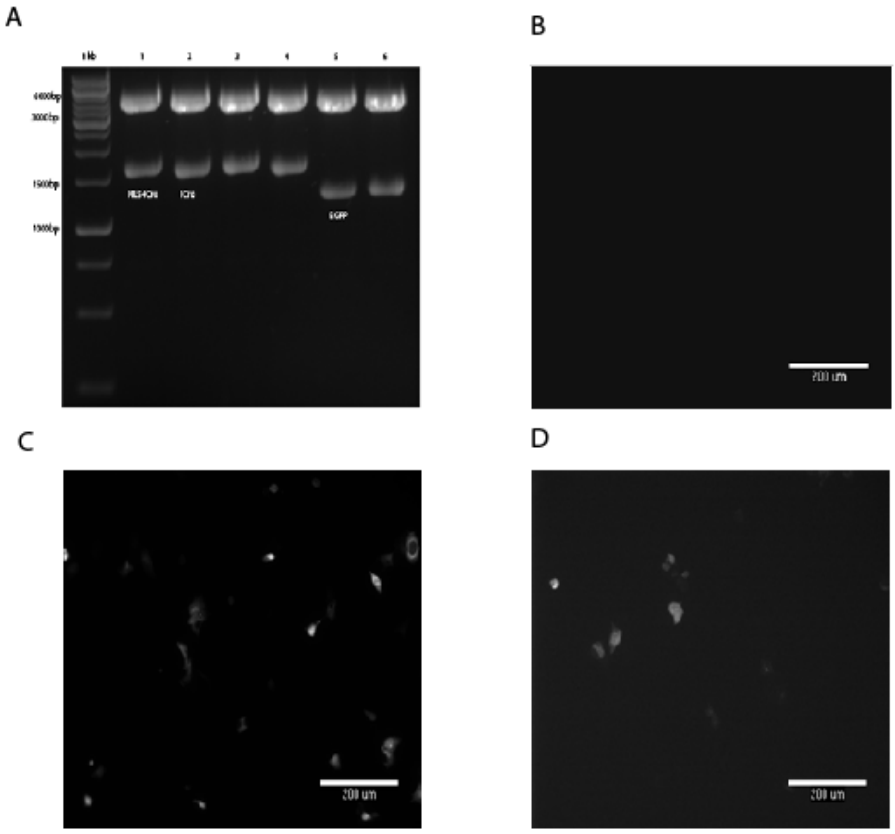


Figure 38 I Verification of CMV-I cam-EGFP-TTC plasmid.

A Control digest, line 6 represents DNA bands corresponding to vector and insert fragment size, B Transfection of HEK293-cell-line and epifluorescence microscopy images, negative control, C as described in B, positive control with functional EGFP-plasmid, C as described in B, test of CMV-I cam-EGFP-TTC plasmid.

The final construct was verified by sequencing the insert and a control digest using restriction endonucleases *Sal*I and *Cla*I with predicted fragments of 1348bp and 4468bp. To test the expression of the fusion protein a HEK293-cell-line was transfected with the CMV-I cam-EGFP-TTC plasmid. Cells were incubated for 24h to allow EGFP-expression. As positive control cells were transfected with a control plasmid containing functional EGFP. As negative control served untransfected cells. The expression level was tested by epifluorescence microscopy.

2.3.6 pCMV-EGFP

This control plasmid can be used to identify the electroporated neuron in an RFP reporter mouse line. For this purpose this plasmid could be mixed with a different plasmid before electroporation. If this construct works, EGFP fluorescence should be visible in the electroporated neuron.

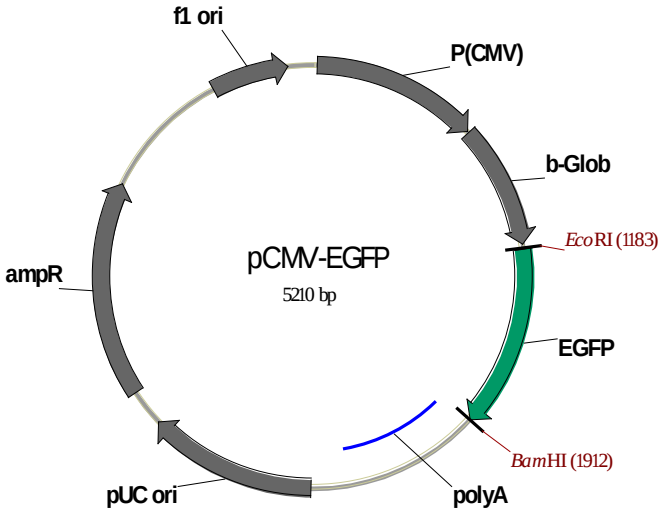


Figure 39 I Vector map of CMV- EGFP plasmid.

pUC ori: E. *coli* origin of replication; ampR: ampicilin resitance gene; ori: f1 phage origin of replication allows the rescue of antisense ssDNA by a helper phage; P(CMV): human cytomegalovirus immediate early promoter for mammalian expression; b-Glob: β-globin gene; EGFP: enhanced green fluorescent protein; polyA: polyadenylation signal for mammalian expression.

The EGFP-sequence (723bp) was cloned into multiple cloning site (MCS) downstream of a 0.4-kb cytomegalovirus (CMV) promoter of the CMV-MCS plasmid (Stratagene).

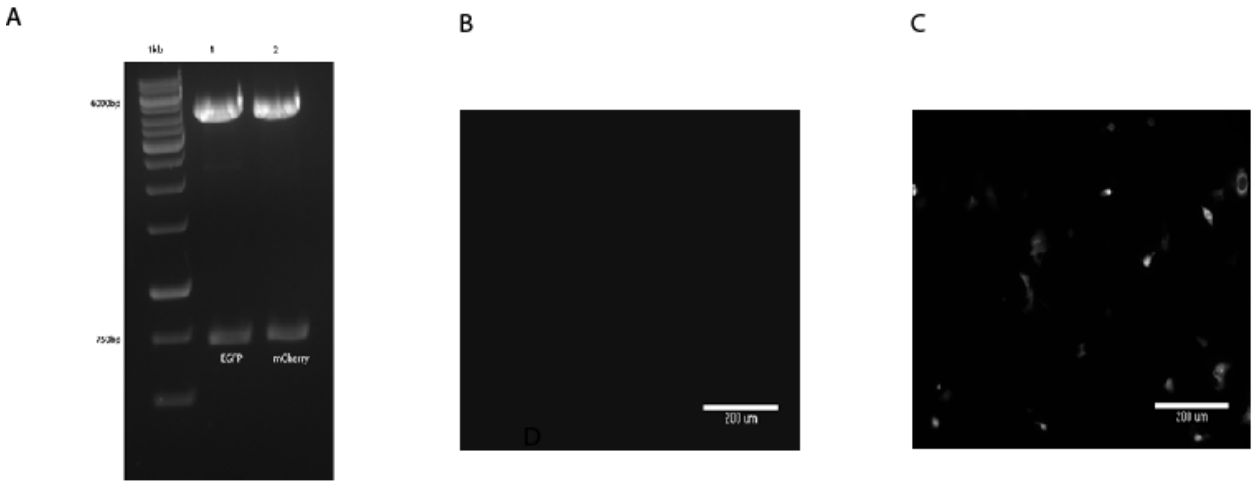


Figure 40 I Verification of CMV-EGFP plasmid.

A Control digest, line 1 represents DNA bands corresponding to vector and insert fragment size, B Transfection of HEK293-cell-line and epifluorescence microscopy images, negative control, C as described in B, functional EGFP-plasmid.

The final construct was verified by sequencing the insert and a control digest using restriction endonucleases *Eco*RI and *Bam*HI with predicted fragments of 723bp. To test the expression of the control plasmid a HEK293-cell-line was transfected with the CMV-EGFP plasmid. As negative control served untransfected cells. Cells were incubated for 24h to allow EGFP expression. The expression level was tested by epifluorescence microscopy.

2.3.7 pCMV-mCherry

This control plasmid can be used to identify the electroporated neuron in a GFP/YFP reporter mouse line. For this purpose this plasmid could be mixed with a different plasmid before electroporation. If this construct works, red fluorescence should be visible in the electroporated neuron.

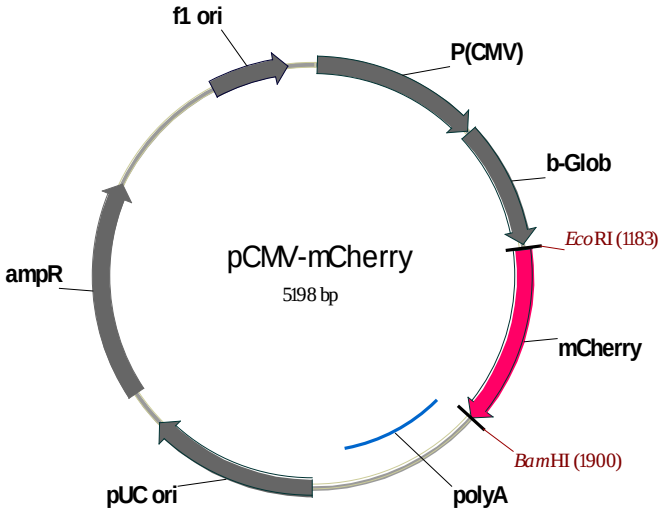


Figure 41 I Vector map of CMV- mCherry plasmid.

pUC ori: E. *coli* origin of replication; ampR: ampicilin resitance gene; ori: f1 phage origin of replication allows the rescue of antisense ssDNA by a helper phage; P(CMV): human cytomegalovirus immediate early promoter for mammalian expression; b-Glob: β-globin gene; mCherry: red fluorescent protein; polyA: polyadenylation signal for mammalian expression.

The mCherry sequence (711bp) was cloned into multiple cloning site (MCS) downstream of a 0.4-kb cytomegalovirus (CMV) promoter of the CMV-MCS plasmid (Stratagene).

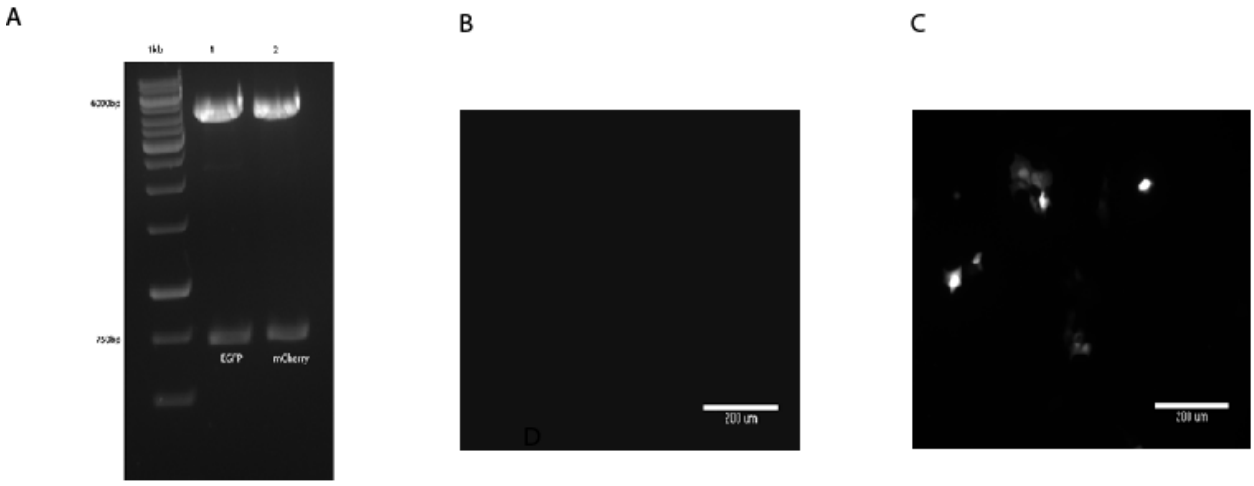


Figure 42 I Verification of CMV-mCherry plasmid.

A Control digest, line 2 represents DNA bands corresponding to vector and insert fragment size, B Transfection of HEK293-cell-line and epifluorescence microscopy images, negative control, C as described in B, functional mCherry-plasmid.

The final construct was verified by sequencing the insert and a control digest using restriction endonucleases *Eco*RI and *Bam*HI with predicted fragments of 711bp and 4487bp for. To test the expression of the control plasmid a HEK293-cell-line was transfected with the CMV-mCherry plasmid. As negative control served untransfected cells. Cells were incubated for 24h to allow mCherry expression. The expression level was tested by epifluorescence microscopy.

2.4 Discussion

The aim of this part of the thesis was to design and to construct expression vectors that would allow the expression of variants of a fusion protein of TTC and Cre-recombinase in order to test the trans-synaptic transport and labeling efficacy. We produced four variants of iCre-TTC-fusion protein constructs, two variable EGFP-TTC constructs previously described in Maskos et al. and two control plasmids for single cell electroporation experiments. They were verified by sequencing and a control digest and finally tested for expression in HEK293-cell-lines using epifluorescence microscopy.

In principle the fusion of two proteins could cause a loss of function of one or both protein parts through structural interference. Therefore we tested the function of Cre-recombinase fused to TTC with transfection of a HEK293-reporter-cell-line carrying a reporter plasmid with GFP and preceding loxP-flanked STOP region. If Cre-recombination would occur, fluorescence should be visible. 24 hours after transfection we could observe fluorescence in transfected cells, indicating that Cre-recombination indeed occurred. However, we observed only low numbers of transfected cells with relatively weak fluorescence, which could potentially indicate impaired function of the fusion protein. Since similar levels of fluorescence had been observerd also in the positive control, a functional Cre-plasmid, we supposed that this finding is rather caused by only low levels of the reporter plasmid in the HEK293-reporter-cell-line. In order to test trans-synaptic transfer and function of the TTC part of the fusion protein our collaboration partners will perform single cell electroporation in neuronal cell culture. Therefore, at first the quality of the reporter mouse-line needs to be tested. A functional Cre-plasmid will be electroporated and subsequent Cre-recombination will allow estimation of the expected level of fluorescence.

In order to identify synaptically coupled neurons using single cell electroporation it would be essential to distinguish between the actually electroporated neuron and the potentially many that receive the fusion protein. For that we produced two plasmids CMV-EGFP and CMV-mCherry which could be used in different reporter mouse lines. One of these plasmids will be co-electroporated with the fusion protein construct but not trans-synaptically transported. Thus, they will provide us with a primary label of the electroporated neuron. We tested both plasmids for expression in HEK293-cell-lines and could observe sufficient fluorescence signals after 24 hours

transfection. Once the single cell electroporation of the constructs had been performed in neurons the fusion protein Cre-TTC will be expressed in the target neuron. Subsequently Cre-TTC is potentially translocated to the nucleus for recombination leading to expression of the fluorescent reporter as a secondary label .

How can the different constructs help us to distinguish potential failure modes of this approach in neuronal cultures? Failure of the fist step, electroporation and expression in the primary neuron, will be detected by a lack of the primary, as well as secondary label. This would be also the case if the cell culture per se would not be viable.

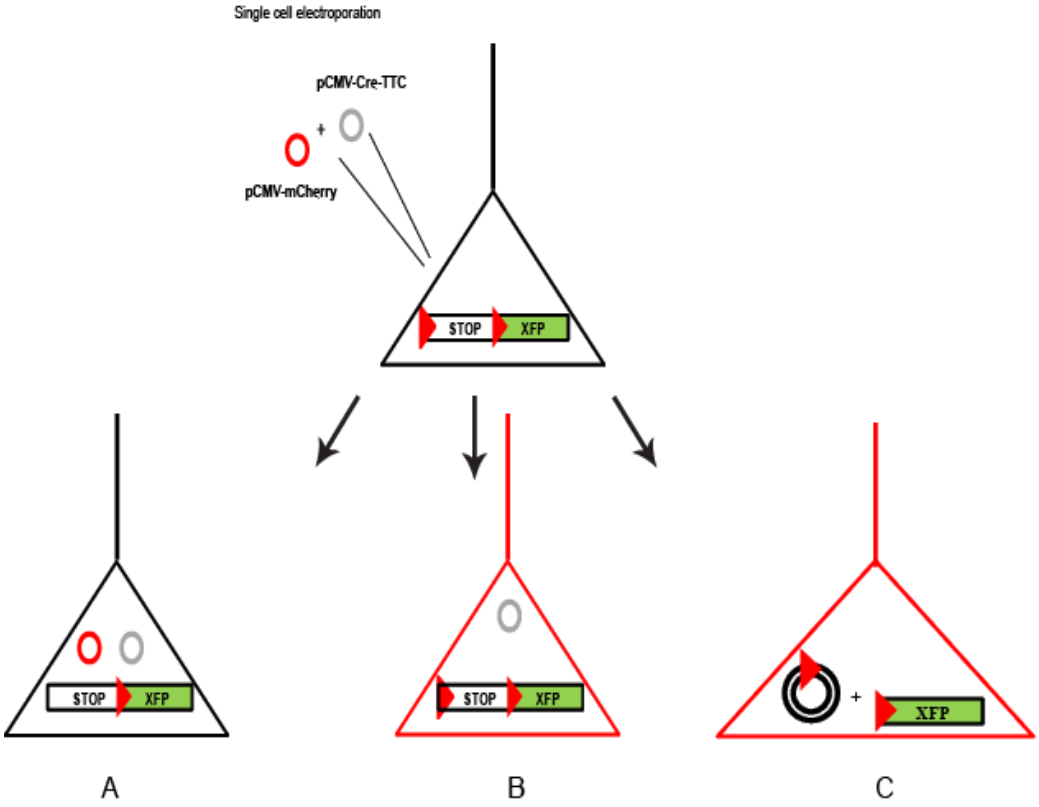


Figure 43 I Single cell electroporation in neuronal cell culture.

The control CMV-mCherry and the CMV-Cre-TTC plasmids are co- electroporatated in the target neuron. A both plasmids are not functional leading to lack of fluorescence B only the control plasmid is functional leading to mCherry fluorescence C Both plasmids are functional leading to mCherry- and XFP-fluorescence.

In principle the translocation of Cre-TTC to the nucleus could interfere with the trans-synaptic transport. Therefore, we produced constructs containing the Cre-recombinase with and without nuclear signal sequence (NLS): I cam / GluR4-NLS-Cre-TTC and I cam / GluR4-Cre-TTC. If the translocation to the nucleus would interfere with the trans-synaptic transport, the two fusion protein variants lacking the NLS, I cam / GluR4-Cre-TTC, should still be transported to second order neurons as indicated by XFP-fluorescence in the electroporated neuron as well as in synaptically coupled neurons.

In general, after translation of membrane associated proteins at the rER they need to be further directed into the membrane trafficking pathway. Naturally short signal peptide sequences, ER export motifs and membrane trafficking signals facilitate this targeted export. Recently, it was shown that the addition of such signal sequences to fusion proteins could enhance their membrane targeting process (Gradinaru, Zhang et al.). Therefore, we considered the two signal peptide sequences I cam and GluR4 could also faciliate the transport of the Cre-TTC fusion protein along the exocytotic pathway: I cam +/-NLS-Cre-TTC and GluR4+/-NLS-Cre-TTC. This would be essential for the further trans-synaptic transport of the fusion protein. If one of the two signal sequences would be better suited we would expect differences in fluorescent signal intensities in synaptically coupled neurons. However, the trans-synaptic transport could generally fail and thus we would only observe mCherry- and XFP- fluorescence in the primary electroporated neuron.

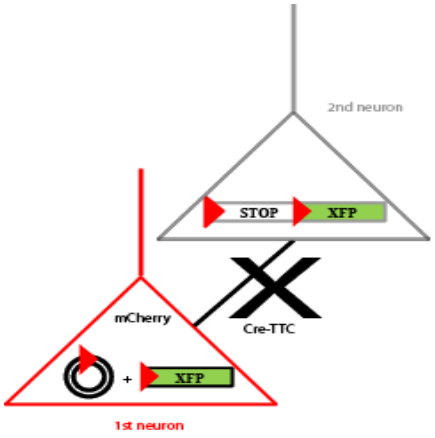


Figure 44 I Failure of trans-synaptic transport of Cre-TTC.

mCherry- and XFP- fluorescence is only visible in the first electroporated neuron.

In the study of Maskos et al. 2007 it was shown that the trans-synaptic transport of GFP-TTC occurred specifically in the retrograde direction. To verify these results, we produced the same EGFP-TTC fusion protein constructs. First, we tested EGFP expression of the fusion constructs in HEK293-cell-lines leading to sufficient fluorescent signals. Further single neuron electroporation experiments will show if also the tetanus toxin part of the fusion protein would be functional. If EGFP fluorescence will be visible in the electroporated neurons as well as in presynaptic neurons the TTC-mediated specific retrograde transport could be verified. Since TTC would be responsible for retrograde trans-synaptic transport this could also lead to multi-synaptic transport to higher order neurons. In this case we would expect XFP-fluorescent signal not only in second order neurons but also in higher order neurons which are not directly connected to the electroporated neuron. This could be tested by targeted electrophysiological recordings that would allow direct estimation of direct synaptic coupling between fluorescent neurons. However, given the fact that neurons typically receive inputs of thousands of neurons the dilution of the transsynaptically transported Cre-TTC beyond secondary neurons will be likely occurring only at extremely low levels.

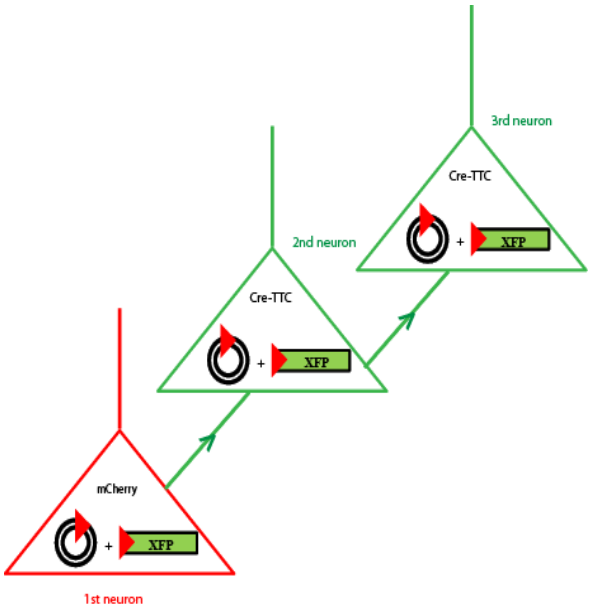


Figure 45 I Possible multi-trans-synaptic transport of Cre-TTC to higher order neurons.

After trans-synaptic transport of Cre-TTC to the directly interconnected second neuron it is further transported to higher order neurons leading to additional fluorescent signals.

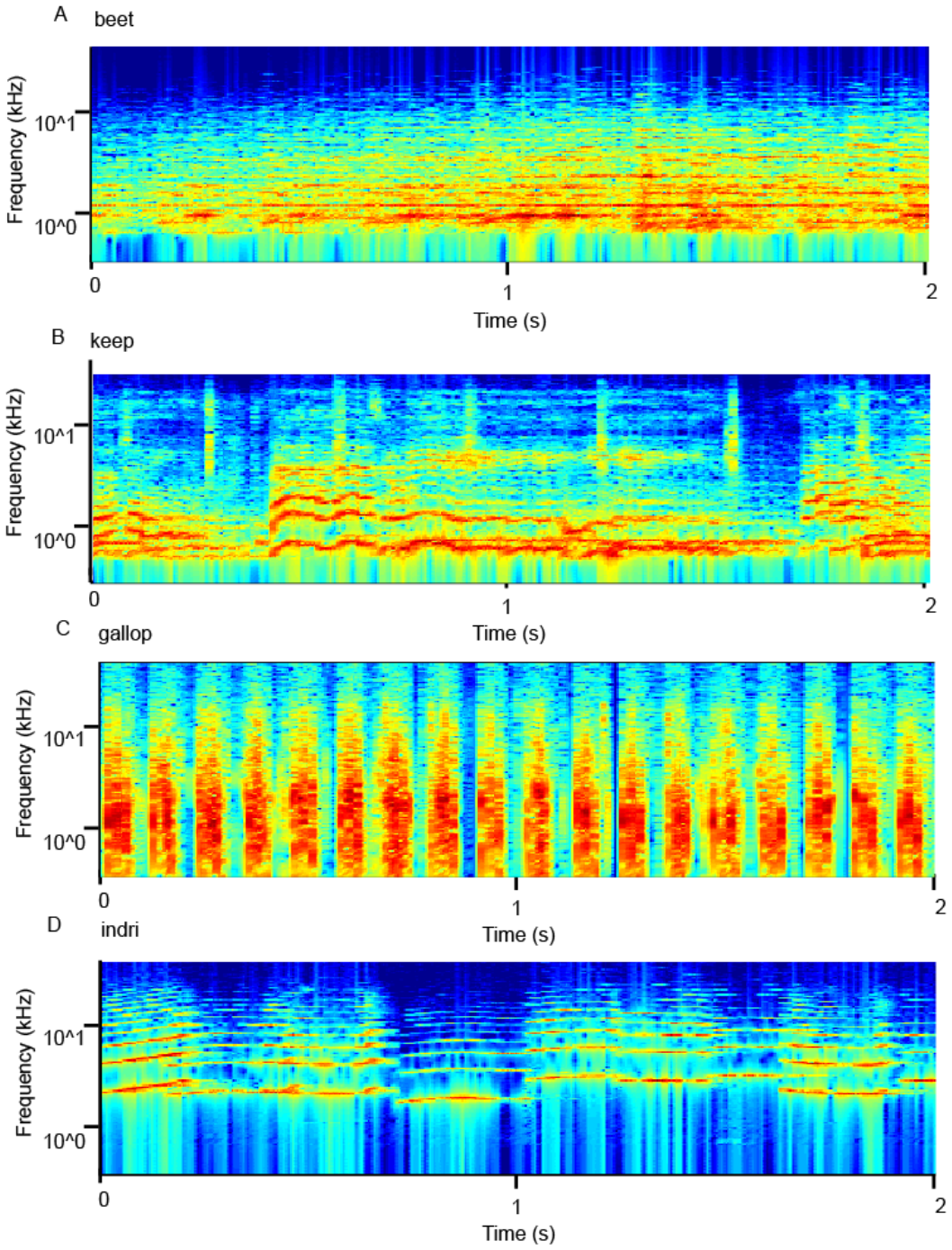
Currently all produced constructs are tested for single cell electroporation in neuronal cell culture by our cooperation partner Dr. Volkmar Lessmann at the University of Magdeburg.

2.5 **Outlook**

The specific labeling of inputs to one single neuron could have multiple advantages in various experimental approaches. In our lab we perform multiple patch clamp recordings in acute brain slices in order to define the connectivity of neurons within specific layers of the auditory cortex. Combining this approach with the retrograde labeling of input neurons would allow us to do paired recordings of synaptically coupled neurons in a targeted fashion and no longer blindly relying on the low average connection probability. Additionally, for a better understanding of auditory cortical functions it is important to observe the activity of large neural populations *in vivo*. Therefore, we perform *in vivo* two-photon calcium imaging which allows us to monitor simultaneously the activity of neuronal networks with single cell resolution. However, currently we have no information about the connectivity within the observed network. It would be for example interesting to know if neurons that are active at the same time show also an exceptionally high probability of being interconnected. A combination of two-photon calcium imaging with single cell electroporation of Cre-TTC in a reporter mouse line could potentially answer this question. This would tremendously improve our current understanding of actual, *in vivo* network processing in the auditory cortex.

3. Appendix

3.1 Sound list



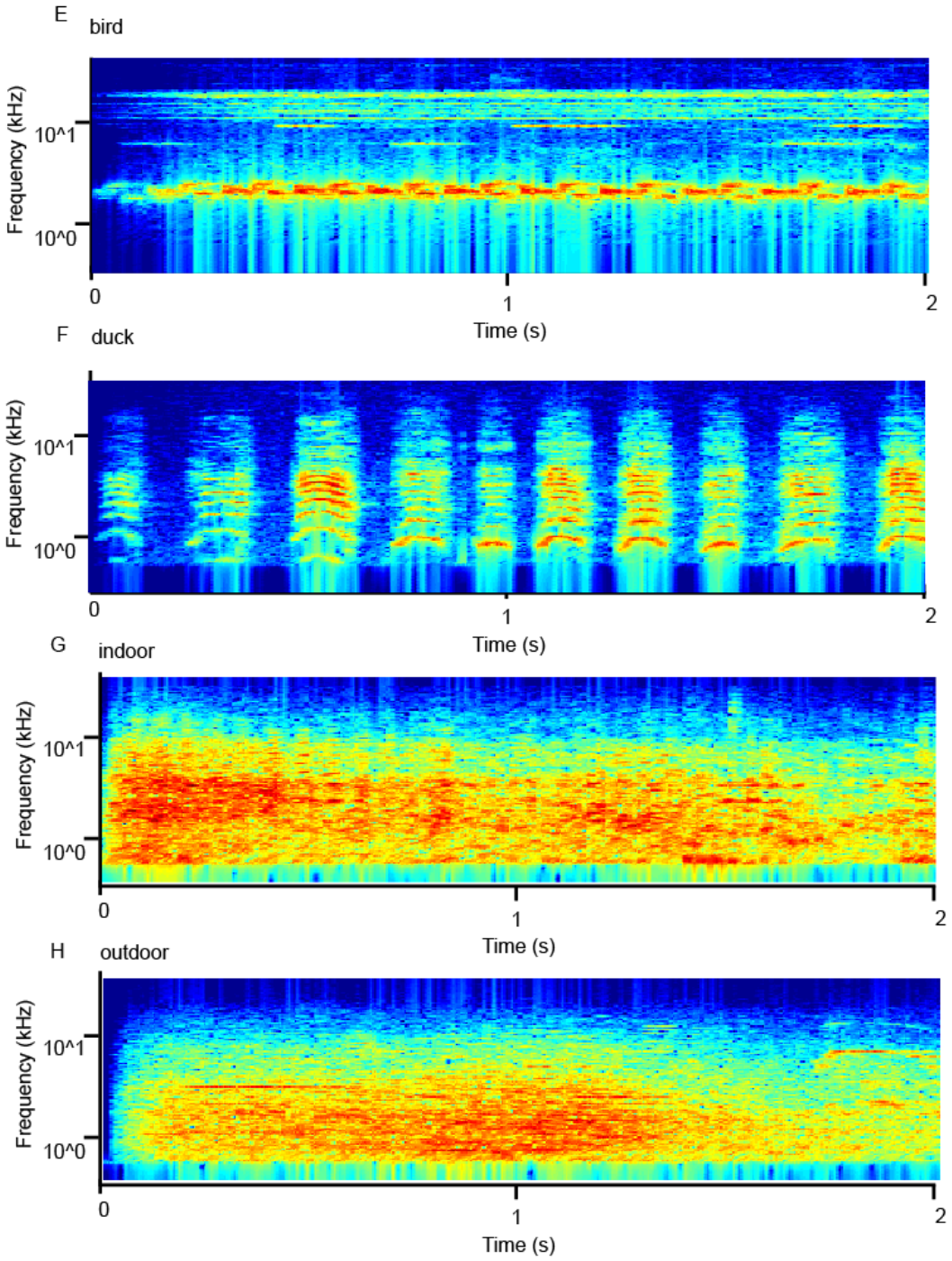


Figure 46 I **Frequency distribution of complex sounds used for classical and operant conditioning.** A beet, B keep, C gallop, D indri, E bird, F duck, G indoor, H outdoor.

3.2. Oligonucleotide List

Tetanus heavy chain

TTC fw1 tctctagacggactctctatgtatttgacaaatgcgccatcgtatactaacggaaaatt

TTC fw2 gaatatatattatagaaggttatataatggactaaaatttattataaaaagatatac

TTC fw3 cctaataatgaaatagattcttttgttaaatcaggtgattttattaaattatatg

TTC fw4 catataacaataatgagcacattgtaggttatccgaaagatggaaatgcctttaataatc

TTC fw5 gatagaattctaagagtaggttataatgccccaggtatccctctttataaaaaaatg

TTC fw6 gcagtaaaattgcgtgatttaaaaacctattctgtacaacttaaattatatg

TTC fw7 gataaaaatgcatctttaggactagtaggtacccataatggtcaaataggcaacg

TTC fw8 ccaaatagggatatattaattgcaagcaactggtactttaatcatttaaaagataaaattttagg

TTC fw9 ataaaattttaggatgtgattggtactttgtacctacagatgaaggatggac

TTC rv1 cggagtcgactcaatcatttgtccatccttcatctgtaggtacaaa

TTC rv2 gtaccaatcacatcctaaaattttatcttttaaatgattaaa

TTC rv3 cattatgggtacctactagtcctaaagatgcatttttatcatcatataatttaagttgtacag

TTC rv4 ggtttttaaatcacgcaattttactgcttccatttttttataaagagggatacctgg

TTC rv5 attataacctactcttagaattctatcaagattattaaaggcatttccatctttcggataac

TTC rv6 caatgtgctcattattgttatatgatacatataatttaataaaatcacctgatttaacaaaag

TTC rv7 ctatttcattattaggtgtatatctttttataataaattttagtccattatataac

TTC rv8 ctataatatatattcaattttccgttagtatacgatggcgcatttgtcaaatacatagagag

I cam

fw

cgtatcgataccatggctcccagcagcccccggcccgcgctgcccgcactcctggtcctgctcggggctctgttcccaggacctggcaatg
cccagacatctgtgtccggatccttt

rv

aaaggatccggacacagatgtctgggcattgccaggtcctgggaacagagccccgagcaggaccaggagtgcgggcagcgcgggcc
gggggctgctgggagccatggtatcgatacg

GluR4

fw

cggatcgataccatgaggattatttgcaggcagattgtcttgttgttttctggattttggggactcgccatgggaggatcccaa

rv

ttgggatcctcccatggcgagtccccaaaatccagaaaacaacaagacaatctgcctgcaaataatcctcatggtatcgatccg

3.3. Primers

iCre

BamHI_NLS_iCre_fw ggtcggatccatggtgcccaagaagaagag

BamHI_iCre_fw ggtcggatccatgtccaacctgctgactgtg

iCre_XbaI_rv ggatctagagacttgtagtccccatcctcgag

EGFP

Ligation with TTC

EGFP_BamHI_fw aattcggatccaccatggtgagcaagggcgag

EGFP_XbaI_rv caaatacatagagagtccgtctagattacttgtacagctcgtc

mCherry/EGFP ligation with pCMV

EGFP_mCherry_EcoRI_fw tcagaattcaccatggtgagcaagggcgag

EGFP_mCherry_BamHI_rv gtaggatccttacttgtacagctcgtccatgc

3.4. Full length sequences

Nuclear Localization Sequence

cccaagaagaagaggaaagtc

I cam (Maskos, Kissa et al. 2002)

atggctcccagcagcccccggcccgcgctgcccgcactcctggtcctgctcggggctctgttcccaggacctggcaatgcccagacatct
gtgtcc

GluR4 (Maskos, Kissa et al. 2002)

atgaggattatttgcaggcagattgtcttgttgttttctggattttggggactcgccatggga

Tetanus Heavy Chain (Dixit, Alam et al. 2006)

atgtatttgacaaatgcgccatcgtatactaacggaaaattgaatatatattatagaaggttatataatggactaaaatttattataaaaagatatac
acctaataatgaaatagattcttttgttaaatcaggtgattttattaaattatatgtatcatataacaataatgagcacattgtaggttatccgaaaga
tggaaatgcctttaataatcttgatagaattctaagagtaggttataatgccccaggtatccctctttataaaaaaatggaagcagtaaaattgcg
tgatttaaaaacctattctgtacaacttaaattatatgatgataaaaatgcatctttaggactagtaggtacccataatggtcaaataggcaacgat
ccaaatagggatatattaattgcaagcaactggtactttaatcatttaaaagataaaattttaggatgtgattggtactttgtacctacagatgaag
gatggacaaatgat

GluR4-NLS-iCre-TTC

accatgaggattatttgcaggcagattgtcttgttgttttctggattttggggactcgccatgggaggatccatggtgcccaagaagaagagga
aagtctccaacctgctgactgtgcaccaaaacctgcctgccctccctgtggatgccacctctgatgaagtcaggaagaacctgatggacatg
ttcagggacaggcaggccttctctgaacacacctggaagatgctcctgtctgtgtgcagatcctgggctgcctggtgcaagctgaacaaca
ggaaatggttccctgctgaacctgaggatgtgagggactacctcctgtacctgcaagccagaggcctggctgtgaagaccatccaacagc
acctgggccagctcaacatgctgcacaggagatctggcctgcctcgcccttctgactccaatgctgtgtccctggtgatgaggagaatcaga
aaggagaatgtggatgctggggagagagccaagcaggccctggcctttgaacgcactgactttgaccaagtcagatccctgatggagaac
tctgacagatgccaggacatcaggaacctggccttcctgggcattgcctacaacaccctgctgcgcattgccgaaattgccagaatcagagt
gaaggacatctcccgcaccgatggtgggagaatgctgatccacattggcaggaccaagaccctggtgtccacagctggtgtggagaagg
ccctgtccctgggggttaccaagctggtggagagatggatctctgtgtctggtgtggctgatgaccccaacaactacctgttctgccgggtca
gaaagaatggtgtggctgccccttctgccacctcccaactgtccacccgggccctggaagggatctttgaggccacccaccgcctgatctat

ggtgccaaggatgactctgggcagagatacctggcctggtctggccactctgccagagtgggtgctgccagggacatggccagggctgg
tgtgtccatccctgaaatcatgcaggctggtggctggaccaatgtgaacatagtgatgaactacatcagaaacctggactctgagactgggg
ccatggtgaggctgctcgaggatggggactacaagtctctagacggactctctatgtatttgacaaatgcgccatcgtatactaacggaaaat
tgaatatatattatagaaggttatataatggactaaaatttattataaaaagatatacacctaataatgaaatagattcttttgttaaatcaggtgattt
tattaaattatatgtatcatataacaataatgagcacattgtaggttatccgaaagatggaaatgcctttaataatcttgatagaattctaagagtag
gttataatgccccaggtatccctctttataaaaaaatggaagcagtaaaattgcgtgatttaaaaacctattctgtacaacttaaattatatgatgat
aaaaatgcatctttaggactagtaggtacccataatggtcaaataggcaacgatccaaatagggatatattaattgcaagcaactggtacttta
atcatttaaaagataaaattttaggatgtgattggtactttgtacctacagatgaaggatggacaaatgattga

GluR4-iCre-TTC

accatgaggattatttgcaggcagattgtcttgttgttttctggattttggggactcgccatgggaggatccatggtgtccaacctgctgactgtg
caccaaaacctgcctgccctccctgtggatgccacctctgatgaagtcaggaagaacctgatggacatgttcagggacaggcaggccttct
ctgaacacacctggaagatgctcctgtctgtgtgcagatcctgggctgcctggtgcaagctgaacaacaggaaatggttccctgctgaacct
gaggatgtgagggactacctcctgtacctgcaagccagaggcctggctgtgaagaccatccaacagcacctgggccagctcaacatgctg
cacaggagatctggcctgcctcgcccttctgactccaatgctgtgtccctggtgatgaggagaatcagaaaggagaatgtggatgctgggg
agagagccaagcaggccctggcctttgaacgcactgactttgaccaagtcagatccctgatggagaactctgacagatgccaggacatca
ggaacctggccttcctgggcattgcctacaacaccctgctgcgcattgccgaaattgccagaatcagagtgaaggacatctcccgcaccga
tggtgggagaatgctgatccacattggcaggaccaagaccctggtgtccacagctggtgtggagaaggccctgtccctgggggttaccaa
gctggtggagagatggatctctgtgtctggtgtggctgatgaccccaacaactacctgttctgccgggtcagaaagaatggtgtggctgccc
cttctgccacctcccaactgtccacccgggccctggaagggatctttgaggccacccaccgcctgatctatggtgccaaggatgactctgg
gcagagatacctggcctggtctggccactctgccagagtgggtgctgccagggacatggccagggctggtgtgtccatccctgaaatcatg
caggctggtggctggaccaatgtgaacatagtgatgaactacatcagaaacctggactctgagactggggccatggtgaggctgctcgag
gatggggactacaagtctctagacggactctctatgtatttgacaaatgcgccatcgtatactaacggaaaattgaatatatattatagaaggtt
atataatggactaaaatttattataaaaagatatacacctaataatgaaatagattcttttgttaaatcaggtgattttattaaattatatgtatcatata
acaataatgagcacattgtaggttatccgaaagatggaaatgcctttaataatcttgatagaattctaagagtaggttataatgccccaggtatc
cctctttataaaaaaatggaagcagtaaaattgcgtgatttaaaaacctattctgtacaacttaaattatatgatgataaaaatgcatctttaggact
agtaggtacccataatggtcaaataggcaacgatccaaatagggatatattaattgcaagcaactggtactttaatcatttaaaagataaaatttt
aggatgtgattggtactttgtacctacagatgaaggatggacaaatgattga

Icam-NLS-iCre-TTC

accatggctcccagcagcccccggcccgcgctgcccgcactcctggtcctgctcggggctctgttcccaggacctggcaatgcccagaca
tctgtgtccggatccatggtgcccaagaagaagaggaaagtctccaacctgctgactgtgcaccaaaacctgcctgccctccctgtggatgc
cacctctgatgaagtcaggaagaacctgatggacatgttcagggacaggcaggccttctctgaacacacctggaagatgctcctgtctgtgt
gcagatcctgggctgcctggtgcaagctgaacaacaggaaatggttccctgctgaacctgaggatgtgagggactacctcctgtacctgca
agccagaggcctggctgtgaagaccatccaacagcacctgggccagctcaacatgctgcacaggagatctggcctgcctcgcccttctga
ctccaatgctgtgtccctggtgatgaggagaatcagaaaggagaatgtggatgctggggagagagccaagcaggccctggcctttgaacg
cactgactttgaccaagtcagatccctgatggagaactctgacagatgccaggacatcaggaacctggccttcctgggcattgcctacaaca
ccctgctgcgcattgccgaaattgccagaatcagagtgaaggacatctcccgcaccgatggtgggagaatgctgatccacattggcagga
ccaagaccctggtgtccacagctggtgtggagaaggccctgtccctgggggttaccaagctggtggagagatggatctctgtgtctggtgt
ggctgatgaccccaacaactacctgttctgccgggtcagaaagaatggtgtggctgccccttctgccacctcccaactgtccacccgggcc
ctggaagggatctttgaggccacccaccgcctgatctatggtgccaaggatgactctgggcagagatacctggcctggtctggccactctg
ccagagtgggtgctgccagggacatggccagggctggtgtgtccatccctgaaatcatgcaggctggtggctggaccaatgtgaacatag
tgatgaactacatcagaaacctggactctgagactggggccatggtgaggctgctcgaggatggggactacaagtctctagacggactctc
tatgtatttgacaaatgcgccatcgtatactaacggaaaattgaatatatattatagaaggttatataatggactaaaatttattataaaaagatata
cacctaataatgaaatagattcttttgttaaatcaggtgattttattaaattatatgtatcatataacaataatgagcacattgtaggttatccgaaag
atggaaatgcctttaataatcttgatagaattctaagagtaggttataatgccccaggtatccctctttataaaaaaatggaagcagtaaaattgc
gtgatttaaaaacctattctgtacaacttaaattatatgatgataaaaatgcatctttaggactagtaggtacccataatggtcaaataggcaacg
atccaaatagggatatattaattgcaagcaactggtactttaatcatttaaaagataaaattttaggatgtgattggtactttgtacctacagatgaa
ggatggacaaatgattga

Icam- iCre-TTC

accatggctcccagcagcccccggcccgcgctgcccgcactcctggtcctgctcggggctctgttcccaggacctggcaatgcccagaca
tctgtgtccggatccatggtgtccaacctgctgactgtgcaccaaaacctgcctgccctccctgtggatgccacctctgatgaagtcaggaag
aacctgatggacatgttcagggacaggcaggccttctctgaacacacctggaagatgctcctgtctgtgtgcagatcctgggctgcctggtg
caagctgaacaacaggaaatggttccctgctgaacctgaggatgtgagggactacctcctgtacctgcaagccagaggcctggctgtgaa
gaccatccaacagcacctgggccagctcaacatgctgcacaggagatctggcctgcctcgcccttctgactccaatgctgtgtccctggtga
tgaggagaatcagaaaggagaatgtggatgctggggagagagccaagcaggccctggcctttgaacgcactgactttgaccaagtcagat
ccctgatggagaactctgacagatgccaggacatcaggaacctggccttcctgggcattgcctacaacaccctgctgcgcattgccgaaatt
gccagaatcagagtgaaggacatctcccgcaccgatggtgggagaatgctgatccacattggcaggaccaagaccctggtgtccacagct

ggtgtggagaaggccctgtccctgggggttaccaagctggtggagagatggatctctgtgtctggtgtggctgatgaccccaacaactacct
gttctgccgggtcagaaagaatggtgtggctgccccttctgccacctcccaactgtccacccgggccctggaagggatctttgaggccacc
caccgcctgatctatggtgccaaggatgactctgggcagagatacctggcctggtctggccactctgccagagtgggtgctgccagggac
atggccagggctggtgtgtccatccctgaaatcatgcaggctggtggctggaccaatgtgaacatagtgatgaactacatcagaaacctgg
actctgagactggggccatggtgaggctgctcgaggatggggactacaagtctctagacggactctctatgtatttgacaaatgcgccatcg
tatactaacggaaaattgaatatatattatagaaggttatataatggactaaaatttattataaaaagatatacacctaataatgaaatagattctttt
gttaaatcaggtgattttattaaattatatgtatcatataacaataatgagcacattgtaggttatccgaaagatggaaatgcctttaataatcttgat
agaattctaagagtaggttataatgccccaggtatccctctttataaaaaaatggaagcagtaaaattgcgtgatttaaaaacctattctgtacaa
cttaaattatatgatgataaaaatgcatctttaggactagtaggtacccataatggtcaaataggcaacgatccaaatagggatatattaattgca
agcaactggtactttaatcatttaaaagataaaattttaggatgtgattggtactttgtacctacagatgaaggatggacaaatga

GluR4- EGFP-TTC

accatgaggattatttgcaggcagattgtcttgttgttttctggattttggggactcgccatgggaggatccaccatggtgagcaagggcgag
gagctgttcaccggggtggtgcccatcctggtcgagctggacggcgacgtaaacggccacaagttcagcgtgtccggcgagggcgagg
gcgatgccacctacggcaagctgaccctgaagttcatctgcaccaccggcaagctgcccgtgccctggcccaccctcgtgaccaccctga
cctacggcgtgcagtgcttcagccgctaccccgaccacatgaagcagcacgacttcttcaagtccgccatgcccgaaggctacgtccagg
agcgcaccatcttcttcaaggacgacggcaactacaagacccgcgccgaggtgaagttcgagggcgacaccctggtgaaccgcatcgag
ctgaagggcatcgacttcaaggaggacggcaacatcctggggcacaagctggagtacaactacaacagccacaacgtctatatcatggcc
gacaagcagaagaacggcatcaaggtgaacttcaagatccgccacaacatcgaggacggcagcgtgcagctcgccgaccactaccagc
agaacacccccatcggcgacggccccgtgctgctgcccgacaaccactacctgagcacccagtccgccctgagcaaagaccccaacga
gaagcgcgatcacatggtcctgctggagttcgtgaccgccgccgggatcactctcggcatggacgagctgtacaagtaatctagacggact
ctctatgtatttgacaaatgcgccatcgtatactaacggaaaattgaatatatattatagaaggttatataatggactaaaatttattataaaaagat
atacacctaataatgaaatagattcttttgttaaatcaggtgattttattaaattatatgtatcatataacaataatgagcacattgtaggttatccgaa
agatggaaatgcctttaataatcttgatagaattctaagagtaggttataatgccccaggtatccctctttataaaaaaatggaagcagtaaaatt
gcgtgatttaaaaacctattctgtacaacttaaattatatgatgataaaaatgcatctttaggactagtaggtacccataatggtcaaataggcaa
cgatccaaatagggatatattaattgcaagcaactggtactttaatcatttaaaagataaaattttaggatgtgattggtactttgtacctacagat
gaaggatggacaaatgattga

Icam-EGFP-TTC

accatggctcccagcagcccccggcccgcgctgcccgcactcctggtcctgctcggggctctgttcccaggacctggcaatgcccagaca
tctgtgtccggatccaccatggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagctggacggcgacgtaaac
ggccacaagttcagcgtgtccggcgagggcgagggcgatgccacctacggcaagctgaccctgaagttcatctgcaccaccggcaagct
gcccgtgccctggcccaccctcgtgaccaccctgacctacggcgtgcagtgcttcagccgctaccccgaccacatgaagcagcacgactt
cttcaagtccgccatgcccgaaggctacgtccaggagcgcaccatcttcttcaaggacgacggcaactacaagacccgcgccgaggtga
agttcgagggcgacaccctggtgaaccgcatcgagctgaagggcatcgacttcaaggaggacggcaacatcctggggcacaagctgga
gtacaactacaacagccacaacgtctatatcatggccgacaagcagaagaacggcatcaaggtgaacttcaagatccgccacaacatcga
ggacggcagcgtgcagctcgccgaccactaccagcagaacaccccccatcggcgacggccccgtgctgctgcccgacaaccactacctg
agcacccagtccgccctgagcaaagaccccaacgagaagcgcgatcacatggtcctgctggagttcgtgaccgccgccgggatcactct
cggcatggacgagctgtacaagtaatctagacggactctctatgtatttgacaaatgcgccatcgtatactaacggaaaattgaatatatattat
agaaggttatataatggactaaaatttattataaaaagatatacacctaataatgaaatagattcttttgttaaatcaggtgattttattaaattatatg
tatcatataacaataatgagcacattgtaggttatccgaaagatggaaatgcctttaataatcttgatagaattctaagagtaggttataatgccc
caggtatccctctttataaaaaaatggaagcagtaaaattgcgtgatttaaaaacctattctgtacaacttaaattatatgatgataaaaatgcatc
tttaggactagtaggtacccataatggtcaaataggcaacgatccaaatagggatatattaattgcaagcaactggtactttaatcatttaaaag
ataaaattttaggatgtgattggtactttgtacctacagatgaaggatggacaaatgattga

EGFP

Accatggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagctggacggcgacgtaaacggccacaagttca
gcgtgtccggcgagggcgagggcgatgccacctacggcaagctgaccctgaagttcatctgcaccaccggcaagctgcccgtgccctg
gcccaccctcgtgaccaccctgacctacggcgtgcagtgcttcagccgctaccccgaccacatgaagcagcacgacttcttcaagtccgcc
atgcccgaaggctacgtccaggagcgcaccatcttcttcaaggacgacggcaactacaagacccgcgccgaggtgaagttcgagggcg
acaccctggtgaaccgcatcgagctgaagggcatcgacttcaaggaggacggcaacatcctggggcacaagctggagtacaactacaac
agccacaacgtctatatcatggccgacaagcagaagaacggcatcaaggtgaacttcaagatccgccacaacatcgaggacggcagcgt
gcagctcgccgaccactaccagcagaacaccccccatcggcgacggccccgtgctgctgcccgacaaccactacctgagcacccagtcc
gccctgagcaaagaccccaacgagaagcgcgatcacatggtcctgctggagttcgtgaccgccgccgggatcactctcggcatggacga
gctgtacaagtaa

mCherry

Atggtgagcaagggcgaggaggataacatggccatcatcaaggagttcatgcgcttcaaggtgcacatggagggctccgtgaacggcc
acgagttcgagatcgagggcgagggcgagggccgcccctacgagggcacccagaccgccaagctgaaggtgaccaagggtggcccc
ctgcccttcgcctgggacatcctgtcccctcagttcatgtacggctccaaggcctacgtgaagcaccccgccgacatccccgactacttgaa
gctgtccttccccgagggcttcaagtgggagcgcgtgatgaacttcgaggacggcggcgtggtgaccgtgacccaggactcctccctgca
ggacggcgagttcatctacaaggtgaagctgcgcggcaccaacttcccctccgacggccccgtaatgcagaagaagaccatgggctggg
aggcctcctccgagcggatgtaccccgaggacggcgccctgaagggcgagatcaagcagaggctgaagctgaaggacggcggccact
acgacgctgaggtcaagaccacctacaaggccaagaagcccgtgcagctgcccggcgcctacaacgtcaacatcaagttggacatcacc
tcccacaacgaggactacaccatcgtggaacagtacgaacgcgccgagggccgccactccaccggcggcatggacgagctgtacaagt
aa

4. References

Abraham, N. M., H. Spors, et al. (2004). "Maintaining accuracy at the expense of speed: stimulus similarity defines odor discrimination time in mice." Neuron **44**(5): 865-76.

Andermann, M.L., A.M. Kerlin et al. (2010). "Chronic cellular imaging of mouse visual cortex during operant behavior and pasive viewing." Frontiers in Cellular Neuroscience **4**(3).

Barad, M., A. M. Blouin, et al. (2004). "Like extinction, latent inhibition of conditioned fear in mice is blocked by systemic inhibition of L-type voltage-gated calcium channels." Learn Mem **11**(5): 536-9.

Binz, T. and A. Rummel (2009). "Cell entry strategy of clostridial neurotoxins." J Neurochem **109**(6): 1584-95.

Blair, H. T., G. E. Schafe, et al. (2001). "Synaptic plasticity in the lateral amygdala: a cellular hypothesis of fear conditioning." Learn Mem **8**(5): 229-42.

Boquet, P. and E. Duflot (1982). "Tetanus toxin fragment forms channels in lipid vesicles at low pH." Proc Natl Acad Sci U S A **79**(24): 7614-8.

Bouton, M. E., R. F. Westbrook, et al. (2006). "Contextual and temporal modulation of extinction: behavioral and biological mechanisms." Biol Psychiatry **60**(4): 352-60.

Coen, L., R. Osta, et al. (1997). "Construction of hybrid proteins that migrate retrogradely and transynaptically into the central nervous system." Proc Natl Acad Sci U S A **94**(17): 9400-5.

Debiec, J., V. Doyere, et al. (2006). "Directly reactivated, but not indirectly reactivated, memories undergo reconsolidation in the amygdala." Proc Natl Acad Sci U S A **103**(9): 3428-33.

Dixit, A., S. I. Alam, et al. (2006). "Sequencing and phylogenetic analysis of neurotoxin gene from an environmental isolate of Clostridium sp.: comparison with other clostridial neurotoxins." Arch Toxicol **80**(7): 399-404.

Fanselow, M. S. and J. E. LeDoux (1999). "Why we think plasticity underlying Pavlovian fear conditioning occurs in the basolateral amygdala." Neuron **23**(2): 229-32.

Frey, U. and R. G. Morris (1997). "Synaptic tagging and long-term potentiation." Nature **385**(6616): 533-6.

Gewirtz, J. C. and M. Davis (1997). "Second-order fear conditioning prevented by blocking NMDA receptors in amygdala." Nature **388**(6641): 471-4.

Gewirtz, J. C. and M. Davis (2000). "Using pavlovian higher-order conditioning paradigms to investigate the neural substrates of emotional learning and memory." Learn Mem **7**(5): 257-66.

Gradinaru, V., F. Zhang, et al. "Molecular and Cellular Approaches for Diversifying and Extending Optogenetics." Cell.

Heffner, H. E., G. Koay, et al. (2006). "Behavioral assessment of hearing in mice--conditioned suppression." Curr Protoc Neurosci **Chapter 8**: Unit8 21D.

Herry, C., S. Ciocchi, et al. (2008). "Switching on and off fear by distinct neuronal circuits." Nature **454**(7204): 600-6.

Kerr, J. N. and W. Denk (2008). "Imaging in vivo: watching the brain in action." Nat Rev Neurosci **9**(3): 195-205.

Kilby, N. J., M. R. Snaith, et al. (1993). "Site-specific recombinases: tools for genome engineering." Trends Genet **9**(12): 413-21.

King, A. J. and I. Nelken (2009). "Unraveling the principles of auditory cortical processing: can we learn from the visual system?" Nat Neurosci **12**(6): 698-701.

Lalli, G., S. Bohnert, et al. (2003). "The journey of tetanus and botulinum neurotoxins in neurons." Trends Microbiol **11**(9): 431-7.

LeDoux, J. (2007). "The amygdala." Curr Biol **17**(20): R868-74.

LeDoux, J. E. (2000). "Emotion circuits in the brain." Annu Rev Neurosci **23**: 155-84.

Lorenzetti, F. D., D. A. Baxter, et al. (2008). "Molecular mechanisms underlying a cellular analog of operant reward learning." Neuron **59**(5): 815-28.

Luksys, G., W. Gerstner, et al. (2009). "Stress, genotype and norepinephrine in the prediction of mouse behavior using reinforcement learning." Nat Neurosci **12**(9): 1180-6.

Madisen, L., T. A. Zwingman, et al. "A robust and high-throughput Cre reporting and characterization system for the whole mouse brain." Nat Neurosci **13**(1): 133-40.

Maren, S. (2001). "Neurobiology of Pavlovian fear conditioning." Annu Rev Neurosci **24**: 897-931.

Maren, S. (2005). "Synaptic mechanisms of associative memory in the amygdala." Neuron **47**(6): 783-6.

Maren, S. and G. J. Quirk (2004). "Neuronal signalling of fear memory." Nat Rev Neurosci **5**(11): 844-52.

Martin, S. J., P. D. Grimwood, et al. (2000). "Synaptic plasticity and memory: an evaluation of the hypothesis." Annu Rev Neurosci **23**: 649-711.

Maskos, U., K. Kissa, et al. (2002). "Retrograde trans-synaptic transfer of green fluorescent protein allows the genetic mapping of neuronal circuits in transgenic mice." Proc Natl Acad Sci U S A **99**(15): 10120-5.

Matteoli, M., C. Verderio, et al. (1996). "Synaptic vesicle endocytosis mediates the entry of tetanus neurotoxin into hippocampal neurons." Proc Natl Acad Sci U S A **93**(23): 13310-5.

Montecucco, C., O. Rossetto, et al. (2004). "Presynaptic receptor arrays for clostridial neurotoxins." Trends Microbiol **12**(10): 442-6.

Montecucco, C., G. Schiavo, et al. (2005). "SNARE complexes and neuroexocytosis: how many, how close?" Trends Biochem Sci **30**(7): 367-72.

Nagy, A. (2000). "Cre recombinase: the universal reagent for genome tailoring." Genesis **26**(2): 99-109.

Nagy, A., L. Mar, et al. (2009). "Creation and use of a cre recombinase transgenic database." Methods Mol Biol **530**: 365-78.

Neubert, J. K., C. King, et al. (2008). "Characterization of mouse orofacial pain and the effects of lesioning TRPV1-expressing neurons on operant behavior." Mol Pain **4**: 43.

Neves, G., S. F. Cooke, et al. (2008). "Synaptic plasticity, memory and the hippocampus: a neural network approach to causality." Nat Rev Neurosci **9**(1): 65-75.

O'Reilly, R. C. (2006). "Biologically based computational models of high-level cognition." Science **314**(5796): 91-4.

Ono, K., M. Kudoh, et al. (2006). "Roles of the auditory cortex in discrimination learning by rats." Eur J Neurosci **23**(6): 1623-32.

Paschall, G. Y. and M. Davis (2002). "Second-order olfactory-mediated fear-potentiated startle." Learn Mem **9**(6): 395-401.

Pavlov, I.P. (1927), "Conditioned reflexes: an investigation of the physiological activity of the cerebral cortex". Lectures 1-13.English translation by G.W. Anrep (1927). http://www.ivanpavlov.com

Pellizzari, R., O. Rossetto, et al. (1999). "Tetanus and botulinum neurotoxins: mechanism of action and therapeutic uses." Philos Trans R Soc Lond B Biol Sci **354**(1381): 259-68.

Rinberg, D., A. Koulakov, et al. (2006). "Speed-accuracy tradeoff in olfaction." Neuron **51**(3): 351-8.

Romanski, L. M. and J. E. LeDoux (1992). "Equipotentiality of thalamo-amygdala and thalamo-cortico-amygdala circuits in auditory fear conditioning." J Neurosci **12**(11): 4501-9.

Rumpel, S., J. LeDoux, et al. (2005). "Postsynaptic receptor trafficking underlying a form of associative learning." Science **308**(5718): 83-8.

Rybalko, N., D. Suta, et al. (2006). "Effect of auditory cortex lesions on the discrimination of frequency-modulated tones in rats." Eur J Neurosci **23**(6): 1614-22.

Sara, S. J. (2000). "Retrieval and reconsolidation: toward a neurobiology of remembering." Learn Mem **7**(2): 73-84.

Schafe, G. E., K. Nader, et al. (2001). "Memory consolidation of Pavlovian fear conditioning: a cellular and molecular perspective." Trends Neurosci **24**(9): 540-6.

Shi, S., Y. Hayashi, et al. (2001). "Subunit-specific rules governing AMPA receptor trafficking to synapses in hippocampal pyramidal neurons." Cell **105**(3): 331-43.

Skinner, B.F. (1984)."The Evolution of Behavior."Journal of The Experimental Analysis of Behavior **41**(2):217-221.

Skinner, B.F. (1984)."The Evolution of Behavior."Journal of The Experimental Analysis of Behavior **41**(2):503-515.

Uchida, N. and Z. F. Mainen (2003). "Speed and accuracy of olfactory discrimination in the rat." Nat Neurosci **6**(11): 1224-9.

Vanover, K. E., S. Robledo, et al. (1999). "Pharmacological evaluation of a modified conflict procedure: punished drinking in non-water-deprived rats." Psychopharmacology (Berl) **145**(3): 333-41.

Weinberger, N. M. (2004). "Specific long-term memory traces in primary auditory cortex." Nat Rev Neurosci **5**(4): 279-90.

Wickersham, I. R., D. C. Lyon, et al. (2007). "Monosynaptic restriction of transsynaptic tracing from single, genetically targeted neurons." Neuron **53**(5): 639-47.

Wickersham, I. R., H. A. Sullivan, et al. "Production of glycoprotein-deleted rabies viruses for monosynaptic tracing and high-level gene expression in neurons." Nat Protoc **5**(3): 595-606.

Yoshihara, Y. (2002). "Visualizing selective neural pathways with WGA transgene: combination of neuroanatomy with gene technology." Neurosci Res **44**(2): 133-40.

Curriculum vitae

Personal Data

Name: Tanja Wernle
Date of birth: 16.01.1982, Innsbruck/Austria
Nationality: Austrian

contact: tanja.wernle@imp.ac.at

Education

June 2000: Matura at the 'Akademisches Gymnasium Innsbruck'

October 2000-2007: Student of biology at the University of Innsbruck

October 2007: Bachelor of Science

October 2007 - April 2009: Master student of molecular biology with specialization in neuroscience at the University of Vienna

April 2009 - April 2010 : Diploma position at the Research Institute of Molecular Pathology Vienna (IMP), Research Group of Simon Rumpel