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"Experience dependent trafficking of the CREB regulated transcription co-activator 1 (CRTC1)"

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Contents

Introduction

The central nervous system

The central nervous system (CNS) comprises the brain and the spinal cord. It allows living beings viability due to surveillance and maintenance of necessary body functions, provides a broad range of cognitive abilities and allows to react and interact with the surrounding environments (Trepel 1999, chapter: Entwicklungsgeschichte des Nervensystems). More precisely, the CNS is composed of the cortex cerebri, the Diencephalon, Mesencephalon, Cerebellum, Pons, Medulla oblongata and the medulla spinalis. Specific parts of the CNS are specialized in different tasks e.g. receiving and processing intrinsic information (medulla spinalis) or maintaining essential bodily functions such as respiration or heartbeat (medulla oblongata).

Looking closer into the human brain, it becomes clear that it consists of hundreds of billions neurons and glia cells. These neurons are wired with each other, forming a network of about hundred thousands of kilometers. It produces a complex structure to store and process information as well as to generate appropriate responses to novel stimuli. Neurons comprise processes called dendrites and axons, facilitating the recognition of neuronal activity as well as the transmission of those signals to linked neurons (Kandel, Schwartz and Jessell 1995, chapter: Neuronen und Verhalten).

By focusing on the neocortex, a division into four lobes is commonly applied, comprising areas such as the Broca area, performing specific functions i.e. speaking and understanding speech or the auditory cortex for integrating and processing sounds (Kandel, Schwartz and Jessell 1995, chapter: Neuronen und Verhalten). The neocortex is organized into 6 layers, exhibiting ~20% mostly inhibitory interneurons and ~80 % pyramidal cells, depicting excitatory neurons. Sensory input enters predominantly layer IV of the neocortex. The signal is forwarded to layer III which transmits it to layer II. Layer II/III together produce an output to linked brain regions while the initial signals gets further transmitted to layer V, depicting the main layer for emission of signals from the neocortex. Layer V is also connected to layer VI, which produces a positive feedback loop due to the activation of the thalamus leading to a signal amplification (Mountcastle 1997; Silberberg et al., 2005). This concept tells us how external cues get to their target areas but it is still unclear which and how many cells are involved in memory formation and storage.

Sound processing in the auditory system

The auditory system mediates perception of a broad range of external acoustic cues by translating these sound stimuli into electrical impulses, directing them further into the auditory cortex at the superior temporal lobe were sensation of auditory information takes place. The pathway of the auditory system starts at the external ear which is composed of the pinna, concha and the auditory meatus, capturing sounds from the environment. The sounds get further passed on into the middle ear, consisting of the tympanic membrane, tympanic cavity, the eustachian tube and the ossicels. The ossicels comprise the malleus, incus and stapes and are connected with the tympanic membrane and the oval window of the inner ear. The malleus receives the oscillation of the tympanic membrane and passes them on over the incus and stapes to the oval window. Due to the specific construction of the ossicels the initial pressure/oscillation of the ear drum is increased by a factor of around 200-fold to overcome the impedance in the inner ear. The cochlea in the inner ear complex decomposes acoustical sound waves due to a break down into simple sinusoidal components. Inner and outer hair cells in the organ of corti further transform the initial external generated pressure waves into neural signals. The resulting frequencies, phases and amplitudes are passed on by the auditory nerve fibers to the auditory radiation pathway (Purves et al., 2008 chapter: The auditory system; King and Nelken 2009). The most important structures in this radiation pathway are the superior olive and the medial geniculate complex of the thalamus. These structures decode the incoming sources and generate a transmission of the original input into the primary auditory cortex. The auditory system therefore performs a conversion of external cues into defined patterns of neuronal activity, generating fast auditory responses and therefore allows an organism to respond to novel or complex incoming acoustical cues in a specific behavioral manner (Purves et al.,2008 chapter: The auditory system).

The primary auditory cortex (A1) comprises frequency selective neurons in a topographic arrangement. This array reflects the linear structure of neurons in the cochlea according to their most sensitive frequency (Tramo et al., 2005). Neighboring neurons process and respond similar to a given frequency (Deweese et al., 2008; Rauschecker et al., 2000; Talavage et al., 2004). Besides tuning to specific frequencies, A1 neurons exhibit the ability to react to amplitudes and duration of a sound frequency as well as to filter pitches and process binaural inequalities. After a sound propagates into the auditory cortex (ACx) the spatiotemporal activity pattern of A1 neurons is believed to underlie the perception and ability to recognize and hear a sound (Moore 2001; Nelken 2004; King and Nelken 2009).

The auditory cortex is a highly plastic structure

The ACx is, beside the ability to process sound information, highly plastic. On the level of spine dynamics, it has been shown that associative fearful events can transiently increase the rate of spine formation in the ACx while non-associative events induce a transiently increased rate of spine elimination (Moczulska et al., 2013). Further fear conditioning experiments, combining an aversive event (light foot shock) with a neutral sound stimulus (tone), showed plasticity of A1 neurons in their receptive field by means of a change in their frequency tuning. In particular, a shift in their best frequencies towards the frequency of the unspecific sound, associated with the undergoing unpleasant event was observed. This shift leads to a re-tuning of the STRF for a given neuron, showing a unique learning induced modification of its receptive field (Bakin and Weinberger 1990; Weinberger 2012). Besides increasing responses, down regulations of best frequencies to pre-conditioned stimuli depicting less important information were monitored as well. (Bakin and Weinberger 1990). Further experiments showed that monkeys, trained to a specific tone exhibited a broader representation of that specific frequency in their primary auditory cortex, illustrating a change in the tonotopic map due to presentation of a naive stimulus (Recanzone et al., 1993). It has also been observed that young animals, getting exposed to a recurrent sound frequency lose their acuity of hearing in the auditory cortex on those borders that are close to this overrepresented frequency in the tonotopic map. In contrast, plasticity observed in adults combined with conditioned behaviour showed an enhancement in the auditory map, interrelated with a betterment in behavioural performance (Schreiner 2014). Beside experimental influence on the ACx it has also been shown that after an injury of certain hair cells in the cochlea a broadening and take-over by adjacent sound frequency responding neurons in the primary auditory cortex occurs (Dahmen and King 2007). Changes in the tonotopic map of the auditory cortex can therefore be easily accomplished by many sound diversifications of the surrounding. Plasticity due to aging, release of neuromodulators or associative learning can lead to alterations of tonotopy (Schreiner 2014). Further experiments dealing with experimental extinction in the auditory cortex showed that stronger memories showed a slower extinction phase, after reversal of the trained behavioral paradigm, as well as a larger tonotopic area to the conditioned tone (Weinberger 2012) Based on these and additional experiments, a large amount of evidence for plastic changes in A1 during learning as well as after alteration processes is recognizable. It depicts that A1 neurons have the ability to build and preserve memory traces (Weinberger 2004). Therefore the ACx see is a good model for observing neuronal activity and following induced plasticity due to learning processes.

Fear conditioning

In 1927 Ivan Pavlov introduced a paradigm termed classical conditioning. For that he trained dogs to a specific sound of a bell (conditioned stimulus CS) and presented the animals with food immediately afterwards (unconditioned stimulus US). He could show, after several sessions, an enhanced salivary production of these dogs through the sound of a bell only, due to the acquired association of the bell with the delivered food (Pavlov 1927; Sejnowski 1999). A specific form of classical conditioning is auditory fear conditioning (AFC), were a neutral sound (CS) is followed by an aversive stimulus (US), in form of a mild electrical shock. Animals will associate the CS with the paired US and show defensive responses (i.e. freezing) as in the occurrence of danger. Therefore, fear conditioning protocol is a paradigm, were learning and long term memory is induced, due to the recognition and association of a neutral stimulus and an aversive stimulus. This initiates a conditioned fear response in an animal, leading to a fearful reaction (Davis 1992; Ledoux 2000; Ressler et al., 2002; Keeley et al., 2006). Auditory fear conditioning (AFC) is a widely used behavioural paradigm as much is known about the circuits mediating this behaviour. Paired-AFC produces memory formation in subjects due to an association of a tone (CS), briefly played prior to a shock (US). Retesting of those subjects in a different environment, presenting only the CS will lead to a fear response expressed via a freezing behaviour. An unpaired conditioning protocol, in contrast, depicts a random distribution of tones and shocks over time, therefore subjects are not able to associate these events with each other and will not generate a memory formation. This protocol can serve as a control to paired auditory fear conditioning (Peter et al., 2012).

The auditory system passes input signals (CS, tone) from the auditory cortex and the auditory thalamus to the lateral part of the amygdala. If an unconditioned stimulus (US, foot shock), transferred via the somatosensory cortex and the somatosensory thalamus reaches the amygdala almost simultaneously, an elevated feedback to the conditioned stimulus is generated, due to the formation of an associative memory (Ledoux 2000; Rogan et al., 1997). Moreover the amygdala presents further many connections to different brainstem structures, eliciting various responses like defensive responses due to the experience of fearful events (Ledoux 2000). Besides the amygdala, however, the ACx appears to be important for auditory fear conditioning. Conditioned auditory stimuli are transmitted via two pathways to the amygdala. They originate both in the thalamus and terminate in the lateral nucleus of the amygdala. The indirect, lemniscal pathway starts at the auditory thalamus, goes through the auditory cortex and ends in the amygdala. The direct, non-lemniscal pathway goes directly from the auditory thalamus into the amygdala (Quirk et al., 1997). It has been shown that lesions of both auditory pathways impair auditory fear

conditioning (Romanski and LeDoux 1992). It seems that especially the lemniscal auditory pathway is crucial for discriminative learning, differentiating between alarming and neutral tones, and plays also an important role for fear memory learning. Lesions of the lemniscal pathway generated an induction of memory deficits in animals after conditioning (Antunes and Moita 2010; Boatman and Kim 2006). Lesions of the cortical ACx before or after animals were trained to sound cues lead also to an interference of conditioned responses when retesting those animals (Quirk et al., 1997; Peters et al., 2012). Taken together, the ACx acts as an important structure for mediating and propagating incoming tones as well as storing information about sound cues.

The molecular mechanism of memory formation

In order to generate learning processes, such as remembering a fearful event, synaptic plasticity between neurons is believed to play an important role. An influential synaptic plasticity model was described by Hebb 1949, proposing an enhancement of the pre- and postsynaptic side of neurons, if they are active at the same time (Sejnowski 1999).This hypothesis also includes associative plasticity due to two different stimuli being active at the same time, but displaying differences in their activation strength. If therefore a presynaptic neuron strongly activates its postsynaptic contact neuron and at the same time a second neuron weakly activates the same contact neuron, there will be a strengthening of the weak signal due to the temporal connection with the strong input. This process underlies the learning of a fear conditioning protocol (Blair et al., 2001). Involved in synaptic plasticity and facilitating strengthening of synapses are N-methyl-d-aspartate receptors (NMDARs), acting as coincidence indicators due to their requirement of pre and postsynaptic activity as well as voltage gated calcium channels (VGCCs), leading to the excitation of a neuron. Both receptors open their channels due to activation and are permeable for calcium (Ca^{2+}) (Shinnick-Gallagher et al., 2003; Malenka and Nicoll 1999.) Ca^{2+} influx achieves the activation of the Ca^{2+} / Calmodulin -Dependent Protein Kinase II (CaMKII), involved in the downstream propagation of the signals onto further transcription factors, leading to the acquisition of memories. CaMKII also activate α-amino-3- hydroxy-5-methyl-4-isoxazolepropionic acid type glutamate receptors (AMPARs), generating a rise in synaptic connectivity (Silva 2003, Malinow and Malenka 2002). After pre-synaptic glutamatergic release, postsynaptic Ca^{2+} influx activates CaMKII. CAMKII and protein kinase A (PKA) mediate AMPA receptor trafficking into the postsynaptic membrane, facilitating strengthening of synapses (long term potentiation, LTP). This mechanism is postulated to be a major mechanism for memory storage (Rumpel et al., 2005). Conversely a removal of AMPA receptors, due to Ca^{2+} influx under a specific threshold, facilitated by protein kinase C (PKC) leads to a decrease in AMPA receptors density and therefore to a

reduction in the capability of a synapse (long term depression, LTD) (Bredt and Nicoll 2003; Shepherd and Huganir 2007; Kessels and Malinow 2009). Beside their important involvement in receptor trafficking and acquiring memories, CaMKII, PKA and PKC play a critical role in the consolidation of memory. PKA gets activated via cAMP, a second messenger in the cell, or through an elevated intracellular Ca2+ level, whereas PKC vitalization is mediated only by an increased amount of intracellular Ca2+. They can both produce signaling cascades via phosphorylation of nuclear translocation proteins or relocate themselves (PKA) and generate memory consolidation dependent gene transcription. CaMKII, PKC and PKA can further activate Mitogen Activated Protein Kinase (MAPK), which acts as an essential kinase in establishing memory consolidation (Johansen et al., 2011). Further studies showed also an important involvement of the neuromodulators norepinephrine and dopamine, constituting monoamine transmitters, in producing memory formation (Bailey et al., 2000; Nader and LeDoux 1999). Taken together, neuronal activity initiated by external cues leads due to the release of stress hormones, neurotransmitter or growth factors to binding of those substances and consequentially activation of receptors present at neuronal synapses. The receptors can either open up their channels or activate secondary messengers such as cAMP, leading to depolarization or downstream activation via derivative messengers in a specific neuron. Influx of positive ions such as Ca2+ and activated kinases act on different transcription factors and proteins to facilitate a transcription of genes involved in memory formation and plasticity (Alberini 2009).

The stabilization of acquired memory

To stabilize acquired memory, transient short term memory (STM) can be shifted into stabile long term memory (LTM), via a process termed memory consolidation. LTP exhibits time-wise phases, early LTP and late LTP. Early LTP is generated through a short single stimulus, underlying the production of STM, represented by quickly remembering e.g. a recently heard multi-digit number. Late phase LTP has been proposed to be the underlying mechanism for long term memory, achieved by multiple stimulations, e.g. by frequently repeating a multi-digit number. STM which is only temporary stable can be accomplished by modification of already existing proteins (Kandel 1997). As opposed to STM, LTM requires gene transcription and the translation of novel proteins (Kandel 2001). To induce protein synthesis, the transcription factors *cAMP responsible element binding protein 1* (CREB1) needs to be activated via phosphorylation by specific kinases, as PKA, MAPK or CaMKII (Abel et al., 1997). CREB1 acts as the key protein in learning and memory. It facilitates gene transcription of cyclic AMP responsible element dependent genes (CRE-element),

leading to the transcription of immediate early genes (IEGs) and further proteins necessary to strengthen synaptic conduction and to establishing prolonged memory formation (Alberini 2009).

Immediate early genes and regulative transcription factors

To establish memory formation CREB1 triggers the translation of IEGs and other genes within minutes, represented by a quick and transient expression level of these IEGs after neuronal activation. The IEGs encode proteins such as c-fos, c-jun or jun-B, are activated via neurotropic factors, neurotransmitter or by an increase in intracellular calcium levels and have the ability to interact with members of different transcription factors or to directly induce transcription (Kovács 1998; Hai et al., 1991; Alberini 2009). Several experiments focusing on IEGs showed a fast transient expression under learning conditions. A significant increased expression was observed after inducing stress, presenting novel objects or sounds to the subjects as well as exposing them to new environments. However, many control experiments, involving a non-learning paradigm or a requirement for protein synthesis to respond to the test, exhibited the same expression or no expression of IEGs. It is therefore not clear which exact processes are induced by IEGs but it seems that novel stimuli increases the expression significantly (Tischmeyer and Grimm 1999). Focusing on the role of IEGs in fear conditioning in the ACx it has been shown that IEGs are up regulated after mice undergoing a fear conditioning experiment. However, IEGs also responded globally to all performed experiments including a foot shock. For example, mice exhibiting a memory formation to an associative event compared to a control group of mice which experienced an unpaired conditioning paradigm, showed no differences in their protein expression levels of IEGs. Therefore it was so far not possible to establish a single IEG as indicator of successful associative learning and memory formation induced by paired conditioning (Peter et al., 2012).

Beside IEGs other proteins exhibit similar abilities to promote gene expression. However, their action does not depend on their own expression levels, but on the subcellular trafficking from the cytosol to the nucleus. The forwarding of distantly generated signals, e.g. from the synapse to the nucleus in a cell, depicts an important factor for regulating gene expression, important for synaptic plasticity. The well-studied transcription factor NF-kB translocates into the nucleus after a glutamatergic stimulus, producing gene expression responses to injuries or infections (Ch'ng 2011; Napetschnig 2013). Another example is the transcription factor, AIDA-1, which translocates via Calcium influx into the nucleus, leading to protein synthesis during a prolonged synaptic stimulus (Jordan et al., 2007). These findings depict a rather unspecific regulation of IEGs for several different stimuli but shows also transcription factors acting rather specific after their

activation. The recently discovered transcription factor CRTC1 (earlier known as TORC1) shows also a translocation into the nucleus, dependent on neuronal activity. In the nucleus CRTC1 binds specific to CREB and facilitates L-LTP or promotes memory formation (Conkright et al., 2003; Bittinger et al., 2004; Sekeres et al., 2012).

CREB-regulated transcriptional co-activator

The protein CREB facilitates, due to its phosphorylation at Ser133, gene expression supporting memory formation via the recruitment of the CREB binding protein (CBP) and p300 to CRE-dependent promoters and their following induction of gene transcription. Interestingly, a recent study showed phosphorylation of CREB at Ser133 does not produce a CRE dependent gene expression at all times (Lonze and Ginty 2002). A recently discovered *CREB-regulated transcriptional co-activator 1* (CRTC1), with expression in the cortex, hippocampus and in the striatum, is able to bind independently of the CREB phosphorylation, specifically at the basic leucin zipper (bZIP) DNA binding domain of CREB and seems to be required for CRE dependent gene transcription (Kovács et al.,2007). This binding enhances the interplay between CREB and the transcription initiation factor TFIID subunit 4 (TFIID), leading to the binding of TFIID to the promoter and an enhanced transcription (Conkright al., 2003.) If the interaction of CRTC1 and CREB gets inhibited, an obstruction of CRE driven gene expression and L-LTP maintenance is observed (Zhou et al., 2006). To establish associative memory, coincidence occurrences have to be detected and integrated to produce synaptic strengthening. CRTC1 is able to connect synaptic stimulation and alteration in gene expression due to signal transmission from activated synapses to the nucleus, by detecting rising intracellular cAMP and Ca2+ levels (Ch'ng et al.,2011; Cohen and Greenberg 2008; West et al., 2002). In inactive neurons, CRTC1 binds a 14-3-3 protein at the synapse via a phosphorylation, mediated by the salt inducible kinase 2 (SIK2), preventing the translocation of CRTC1 into the nucleus (Clark et al., 2012; Ravnskjaer et al., 2007; Screaton et al., 2004). The activity of SIK2 in the cytoplasm is regulated by the Ca2+/calmodulin-dependent kinase I/IV (CaMK) (Henriksson et al., 2012). Nuclear translocation of CRTC1 is initiated by synaptic activity and mediated by calcium influx through AMPA, NMDA and L-type voltage-gated calcium channels (LVGCCs) (Ch'ng et al., 2012). Calcineurin, a phosphatase, dephosphorylates CRTC1 after calcium influx through the channels and allows CRTC1 to translocate into the nucleus (Bittinger et al., 2004). A second pathway, allows prolonged arrest of CRTC1 in the nucleus, produced by dopamine or norepinephrine receptor signalling, activating the adenylyl cyclase and guiding a rise the intracellular cAMP level (Kovács et al., 2007). Cyclic AMP activates the protein kinase A (PKA), which blocks a negative feedback loop initiated by CRTC1 itself. CRTC1 induces

this feedback via the gene expression of SIK1, leading to a binding competition of CRTC1 and SIK1 at the bZIP domain of CREB (Katoh et al., 2004; Zhang et al., 2009; Jagannath et al., 2013). CRTC1 is suppressed by SIK1 and translocate back into the cytoplasm. If PKA is activated by cAMP, PKA can block SIK1 and prevent its binding at the bZIP domain, prolonging the nuclear accumulation of CRTC1 (Henriksson et al., 2012).

Due to its two-fold regulation CRTC1 could potentially show more regulatory specificity compared to IEGs. It would therefore be interesting to gain more insight into the regulation and translocation of CRTC1 in the *in vivo* situation. CRTC1 translocation may mark neurons for a specific condition when visualizing it after various behaviour paradigms.

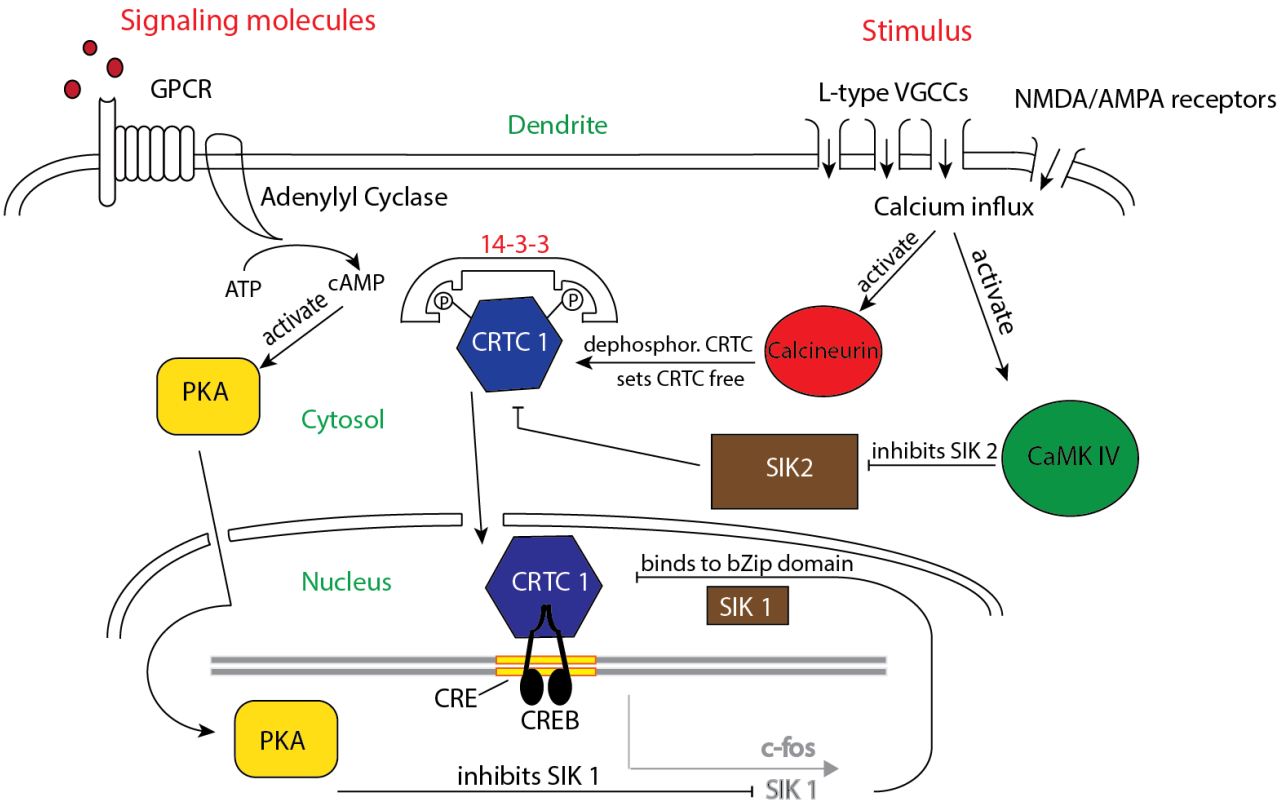


Fig 1) Neuronal activity dependent pathways of CRTC1

After a neuronal stimulus Ca2+ enters the neuron via NMDA/AMPA as well as the L-type VGCCs. The intracellular increase in Ca2+ leads to the activation of Calcineurin and CaMK IV. Due to the dephosporylation of the 14-3-3 protein and the inhibition of SIK2, CRTC1 gets released and translocate into the nucleus were CRTC1 binds at the bZIP domain of CREB. CRTC1 produces a negative feetback loop due to the transcription of SIK1 by CRTC1 itself. SIK1 competes and displaces CRTC1 at the bZIP domain. Cell culture experiments showed a prolonged occurrence of CRTC1 in the nucleus after activation of the Adenylyl cyclase – PKA pathway. PKA inhibits the function of SIK1 and allows an increase in the binding time of CRTC1 with CREB.

In-vivo findings and involvement of CRTC1 in memory formation process

Recent *in vivo* experiments showed after viral-mediated overexpression of CRTC1 in the dentage gyrus of the hippocampus enhanced memory formation after contextual fear conditioning. Beside this increased consolidation response, reconsolidation which requires protein synthesis as well, was also elevated after animals were injected with CRTC1. Interestingly, when inducing a viral-mediated overexpression of CRTC1 after memory acquisition, no elevated fear responses were observed. This leads to the conclusion that CRTC1 promotes fearful memory if the viral-mediated overexpression of CRTC1 was prior to memory acquisition or before memory reconsolidation (Sekeres et al., 2012). Another *in vivo* study showed light exposure dependent relocation of CRTC1 in the murine suprachiasmatic nucleus (SCN). This rhythmic regulation depends on translocation of CRTC1 into the nucleus or/and increased stability of CRTC1 mRNA due to light stimulation. Overexpression of CRTC1 showed an up regulation of period1, a core clock gene, indicating a strong involvement of CRTC1 in the circadian rhythm due to activity dependent translocation of CRTC1 (Sakamoto et al., 2013)

It has been suggested that LTP, facilitating synaptic strengthening, acts as the underlying mechanism for memory storage. Early LTP, depending on modification of pre-existing proteins induces short term alterations of synapses, while late-LTP generating stable and lasting synaptic modifications depends on gene transcription (Cohen and Greenberg 2009). It has been shown that CREB acts as the key regulator for long term memory facilitating gene transcription due to the binding of the cAMP response element (CRE) present in promoters of many target genes (Kandel 2001). *In vitro* experiments showed that CRTC1 translocation and binding to CREB is required and sufficiently for CRE dependent gene expression (Bittinger et al., 2004; Zhou et al., 2006). Further *in vitro* experiments showed CRTC1 activity dependent translocation correlated with the induction of a late-LTP while a CRTC1 knockout completely revoked an induced L-LTP. Taken together, *in vitro* experiments showed that CRTC1 plays an important role in the integration of synaptical produced signals, guiding those signals into the nucleus and facilitate gene expression as well as the generation of an L-LTP leading to memory storage (Zhou et al,. 2006). However, how endogenous CRTC1 may translocate *in vivo* during different behavioural paradigms is unknown.

Central question

Previous in-vitro studies have shown the expression of CRTC1 in primary neuronal cell cultures and its regulated transport from the synapse to the nucleus (Zhou et al., 2006; Kovács et al., 2009; Li et al., 2009; Ch'ng et al., 2012). It is known that the auditory cortex is highly plastic, showing increased display of great spine turnover after exposure of subjects to different associative learning tasks and seem to play a role in memory formation (Moczulska et al., 2013). Therefore the ACx demonstrates a good model to test for a possible translocation of CRTC1 into the nucleus *in vivo* after individuals undergo a fear learning paradigm.

The question of my master thesis reads as following:

Does CRTC show subcellular trafficking during different behaviour paradigms?

To answer the question I established an immunohistochemistry staining in combination with a behaviour paradigm to locate the respective position of CRTC1 *in vivo*.

Materials and Methods

Animals

In all conducted experiments, mice were male, *C57Bl/6J* within an age of 9 to 10 weeks. Mice were all provided by the in-house mouse facility, kept in groups of four and housed in 530cm^2 cages. Mice were kept on a day/night cycle; 12 hour light, from 6 am to 8pm, and 12 hour darkness, from 8pm to 6am. Mice had access to dry food and water as well as a small shelter to hide and sleep in, at all times.

Buffers, solutions and antibodies

Phosphate buffered saline (PBS): 137mM sodium chloride

2,7mM potassium chloride

12mM Phosphate

pH-value: 7,4

Ketamine/Medetomidine stock solution

Ingredient; Volume (ml) and weight (mg/g mouse weight)
Ketamine (AniMedica), 1 ml and 2.5 mg/g
Medetomidinhydrochlorid 1mg (Domitor), 0.8 ml and 0.02 mg/g
Distillated water, 8.2 ml

Primary antibodies

TORC1 (CRTC1) monoclonal rabbit, Cell signaling technology Inc.

CamKII monoclonal mouse, ThermoFisher Scientific Inc.

NeuN polyclonal guinea pig, ©EMD Millipore Corporation

Secondary antibodies	Alexa Flour®, 488 goat anti rabbit antibody, Life technologies ™
	Alexa Flour®, 633 goat anti mouse antibody, Life technologies ™
	Alexa Flour®, 633 goat anti guinea pig antibody, Life technologies™

Perfusion

Mice were deeply anesthetized with 500µl Ketamine/Medetomidine, injected intraperitoneally and put back into their cages for 5-6 minutes. The response after tweaking the mice in the tail/foot was observed. When the absence of any reflex and non-responsibility to the inconvenient stimuli was observed, mice were fixed dorsally with pins 19mmx 0,4mm (BD Microlance™ 3) on the Styrofoam work space. Mice were sliced up from the urinal bladder to the neck by using a small surgical scissor. After opening of the abdomen, the lateral conjunctive tissue was removed to increase the opening of the body cavity. By cutting the breastbone and immediately afterwards removing the diaphragm from the thoracic wall, the thorax was opened to gain access to the heart. The rip-cage was afterwards hanging lose and pinned down laterally beside the mouse. After removing connective tissue around the beating heart, a surgical forceps was used to stabilize and hold the heart while inserting in succession a needle with 21G x3/4”-0,8x19mm (Terumo® Europe N.V.) into the left ventricle. The needle was connected over a tube to a bottle filled with PBS. After insertion of the needle, PBS influx was activated and pumped into the left ventricle. Immediately afterwards the heart was cut open at the right atrium to release the blood and flush the bloodstream with PBS. After the throughput of 20ml PBS the mouse was fixated with 15ml of 4% paraformaldehyde (PFA) (Thermo Fisher Scientific Inc.). The mice were decapitated and the skin over the brain was opened with a scalpel. The skullcap got cut open along the sagittal suture with two small cuts sideward above the front of the forebrain. Brainpan was now removed with a forceps to reach the brain which was pulled out with a shovel-like surgery tool. The brains were immediately put into 12ml of 4 % PFA for the duration of 5 to 7 hours. After this fixation step, brains were stored in PBS for 30 to 40 hours.

Vibratome

Brains were carefully removed from PBS and cut coronal with a blade (Bic Violex S.A.-AG) at the isocortex to remove the olfactory bulb and parts of the cerebral cortex. This process was performed to receive an even plane for fixing the brain anterior with superglue (Carl Roth GmbH+Co_KG). After fixation, the brains were cut with a vibratome, (VT-1000 Leica) starting from

the cerebellum. Coronal sections of 70µm thickness of the auditory cortex were obtained by cutting through the fixed brain. The slices were immediately put into PBS in 24well plates (BD Bioscience) for further immunohistochemistry treatment.

Immunohistochemistry

CRTC1 staining

Slices were blocked at room temperature in PBS containing 1% Triton-X 100 (Sigma/Fluka) and 10% NG22 Normal goat serum (Szabo-Scandic HandelsgmbH & Co KG) for the duration of 2h. Slices were washed 1x with PBS and further incubated with PBS comprising 5% Normal goat serum (NGS), 0, 1% Triton-X 100 and the primary TORC1 (CRTC1) antibody (Cell signaling technology, Inc), 1:5000 diluted, at 4 degree overnight. On the next day, slices were exposed to room temperature for 1h, followed by 3x washing with PBS. PBS containing 5% NGS, 0,1% Triton-X 100 and the secondary Alexa Flour®, 488 goat anti rabbit antibody (Life technologies ™), 1:1000 diluted were applied to the slices and incubated for 3h. After 2 hours and 50 minutes slices were additionally stained with 4', 6-diamidino-2-phenylindole (DAPI, molecular probes – Life technologies ™) diluted 1:5000 in 1xPBS. Subsequently slices were rinsed 3x with PBS and transferred onto objective slides.

CaM Kinase II staining

Calcium/Calmodulin –dependent protein kinase II is a highly abundant serine/threonine kinase in the brain (Lisman et al., 2002). It is part of the postsynaptic density and involved in mediating synaptic plasticity and the regulation of glutamatergic synapses (Liu et al., 2002). It has been shown in several experiments that CaM Kinase II is localized in excitatory neurons in the forebrain, hippocampus or cerebral cortex but not in GAD/GABA positive inhibitory neurons (Benson et al., 1992; Sík et al., 1998 Ochiishi et al., 1994). Therefore anti- CaM Kinase II antibody can be used to identify excitatory neurons.

Slices were blocked at room temperature in PBS containing 1% Triton-X 100 and 10% NGS for 2h. Slices were washed 1x with PBS and incubated with PBS comprising 5%NGS, 0, 1% Triton-X 100 and the primary CaM Kinase II antibody (Thermo Fisher Scientific Inc.), 1:1000 diluted, at 4 degree overnight. On the next day, slices were exposed to room temperature for 1h, followed by 3x washing with PBS. Furthermore PBS containing 5% NGS, 0, 1% Triton-X 100 and the secondary Alexa Flour®, 633 goat anti mouse antibody (Life technologies ™), 1:1000 diluted, were

applied to the slices and incubated for 2h. After 1hour and 50 minutes slices were additionally stained with DAPI, diluted 1:5000 in 1xPBS. Subsequently slices were rinsed 3x with PBS and transferred onto objective slides.

CRTC1 and CaM Kinase II staining

Slices were blocked at room temperature in PBS containing 1% Triton-X 100 and 10% NGS for 2h. Slices were washed 1x with PBS and incubated with PBS comprising 5%NGS, 0, 1% Triton-X 100 and the primary CaM Kinase II (1:1000) and TORC1 (1:5000) antibodies at 4 degree overnight. On the next day, slices were exposed to room temperature for 1h, following 3x washing with PBS. Furthermore PBS containing 5% NGS, 0, 1% Triton-X 100 and the secondary antibodies Alexa Flour®, 633 goat anti mouse (1:1000) and Alexa Flour®, 488 goat anti rabbit (1:1000) were applied to the slices and incubated for 2h. After 2hours and 50 minutes slices were additionally stained with DAPI, diluted 1:5000 in 1xPBS. Subsequently slices were rinsed 3x with PBS and transferred onto objective slides.

NeuN staining

The anti -neuronal nuclei (NeuN)-antibody can bind specific to its antigen located in the nuclei of neurons. NeuN is localized in the majority of neurons in the brain, in the cerebal cortex, cerebellum, hippocampus and further structures as well as in the peripheral nervous system (Mullen et al., 1992). It is a member of the FOX family, a neuron specific RNA binding protein which is important for neuronal differentiation (Kim et al., 2013). The Anti-NeuN antibody is a neuronal specific marker and therefore widely used to identify post mitotic neurons (Kim et al., 2009).

Slices were blocked at room temperature in PBS containing 1% Triton-X 100 and 10% NGS for 2h. Slices were washed 1x with PBS and incubated with PBS comprising 5%NGS, 0, 1% Triton-X 100 and the primary Anti-NeuN antibody (©EMD Millipore Corporation), 1:1000 diluted, at 4 degree overnight. On the next day, slices were exposed to room temperature for 1h, followed by 3x washing with PBS. Furthermore PBS containing 5% NGS, 0, 1% Triton-X 100 and the secondary Alexa Flour®, 633 goat anti guinea antibody (Life technologies ™), 1:1000 diluted, were applied to the slices and incubated for 2h. After 1hour and 50 minutes slices were additionally stained with DAPI, diluted 1:5000 in 1xPBS. Subsequently slices were rinsed 3x with PBS and transferred onto objective slides.

NeuN and CRTC1 staining

Slices were blocked at room temperature in PBS containing 1% Triton-X 100 and 10% NGS for 2h. Slices were washed 1x with PBS and incubated with PBS comprising 5%NGS, 0, 1% Triton-X 100, the primary Anti-NeuN antibody (©EMD Millipore Corporation) (1:1000) and TORC1 (1:5000) antibodies at 4 degree overnight. On the next day, slices were exposed to room temperature for 1h, followed by 3x washing with PBS. Furthermore PBS containing 5% NGS, 0, 1% Triton-X 100 and the secondary Alexa Flour®, 633 goat anti guinea antibody (Life technologies ™), 1:1000 diluted and Alexa Flour®, 488 goat anti rabbit (1:1000), were applied to the slices and incubated for 2h. After 1hours and 50 minutes slices were additionally stained with DAPI, diluted 1:5000. Afterwards slices were rinsed 3x with PBS and transferred onto objective slides.

Mounting and object slides

Brain slices were put on the object slides “Superfrost Ultra plus ®” (Thermo Fisher Scientific Inc.) with a brush. For each brain slice a PBS drop was applied onto the objective slides to enable a transfer of the stained slices from the 24-well plates onto the slides. For fixation, the DAKO mounting medium (Pathology Products Dako Oesterreich GmbH) was applied to mount on the slides after removing the PBS droplet and adjust the brain slices to a horizontal position. Cover slips (Menzel-Glaeser, 24x60x1, 5mm) were slowly laid over the brain slides to enable an analysis via confocal microscopy.

Confocal microscopy

Images of all experiments were obtained with an Axiovert 200M / LSM 500 Zeiss microscope (Carl Zeiss MicroImaging GmbH, Germany). Objective slices were covered with Immersol™ 518F by Zeiss (Carl Zeiss AG) and imaged with a Plan-Neofluar 40x/1, 3 Oil DIC. Lasers used for imaging were: Laser Diode 405, 405nm, Argon/2 with 488nm and HeNe2 633nm. Images of the auditory cortex were tile scanned with 5 vertical and 5 horizontal tiles with 5120 Pixels and a size of 649µm. Images were processed by the ZEN 2008 (Carl Zeiss AG) software. Brain slice acquired from the vibratome (Material and Methods, Vibratome) had a thickness of 70µm. Images of these brain slices were taken at a depth of 20µm for all images in all established stainings.

Analysis of single cells

In the first step of the analysis the software Developer tissue studio was used to determine and map the layers of the cortex. Images of the ACx were overlaid with manually annotated regions of interest (ROI) delineating the ACx into layer 1, layer 2/3, layer 4 and layer 5/6. In the next step of the analysis, Definiens Software Suite was used to generate single cell values to determine the main localization of CRTC1. First, detection of nuclei and segmentation were performed on DAPI in Definiens Tissue Studio, using manually drawn regions of interest representing brain layer 2/3, layer 4 and layer 5/6. Afterwards, Definiens Developer was used to analyse intensities of nuclear and cytoplasmic staining of those regions for CRTC1. For this purpose a ring shaped object was drawn around every nucleus to represent the cytoplasm. Mean intensities of nuclear objects and cytoplasm rings were then evaluated for every cell.

Behaviour chambers

Main equipment:

HP Compaq Computer; Coulbourn Precision Animal shocker; 351-31Bande ART Frequency regulator Infrared camera with IR-lamp CCD KB R3138; Matlab (Mathworks, NATICK); Soundcard: 4Hz to 80 000Hz; loud speakers: frequency area : 1 to 80kHz

Behaviour chambers comprised a metal enclosure which allowed the insertion of artificial environments. The chosen context was a white circular area lubricated with a citrus/water mix. The floor consisted of a grid shaped shocker with a green subsurface composed of a green towel and a green insert able ark. Mice were habituated for 15 minutes from 10:00am to 10:15am for 4 days. Mice were immediately placed back into their home cages. After 4 days of habituation mice were again placed in the noise proofed chambers, completing a fear conditioning protocol.

Fear conditioning

For fear conditioning, 36 male *C57Bl/6J* mice were distributed into 3 groups of 12 mice: home cage, paired and unpaired mice. The first category represented the control group with naive home cage mice, which were left inside their cages until they were sacrificed. The second group, “paired mice” were habituated 4 days for 15 minutes from 10:00am to 10:15am to the experimental environment. On day 5, fear conditioned to a complex tone (Fig 1a, Fig 1b) coupled to an immediately following shock of 0.7mV was done from 10:00am to 13:00pm. The tones played in the chambers covered partially the hearing range of mice. The third group, “unpaired mice” were

also habituated to the experimental setup (Material and Methods; Behaviour chambers) for 4 days. On the next day, mice received, just as the paired mice, a fear conditioning protocol with the exception that shocks and tones were randomly distributed (Fig 1c). After the conditioning session mice were immediately placed back in their cages. One hour after the conditioning process, mice were anesthetized with 500µl of Ketamine/Medetomidine into the peritoneum and prepared for the perfusion.

The software running the fear conditioning protocol for the experimental setup was written in Matlab 7.4.0(R2007) by Dr. Rumpel. For the protocol I used a complex sound with a volume intensity of 200 to 1500 arbitrary units and a maximal sampling frequency of 192 kHz. Data from our lab showed that animals need their ACx to process the applied complex sound and acquire a fearful memory. Mice exhibiting ACx lesions were not able to process this complex sound, indicated by a reduced freezing behaviour (Peter et al., 2012) (Fig 2a).The established protocol, linking the conditioned stimuli (sound) to the unconditioned stimuli (shock) comprised the activation of the house light in the chamber, a shock with 0.7mV for the period of 1 second, elicited by the shocker below the mice and the playing of a complex sound with a length of 2 seconds (Fig 2b). The protocol contained an absolute baseline phase where no shock/sound was played. After a 10 second fixed period followed by a random interval between 1 and 25 seconds, with no application of any stimuli in both cases, a shock coupled to a sound was delivered. Subsequently an after-sound period and a baseline period are activated were no stimulus got delivered. The coupling of the sound and the immediately following shock was 5 times repeated for one session with an interval of 50 to 75 seconds between each occurrence (Fig 2d). The protocol for the unpaired mice comprised the activation of the house light in the chamber, a shock length of 1 second, with an electrical voltage of 0,7mV and a complex sound with the length of 2 seconds (Fig 2c). The complex sound was repeated 5 times but in contrast to the paired protocol there was no coupling of the sound and the shock but a random distribution. After receiving the protocol, paired and unpaired conditioned mice were immediately put back into their cages and after a 1hour waiting period anesthetised and perfused.

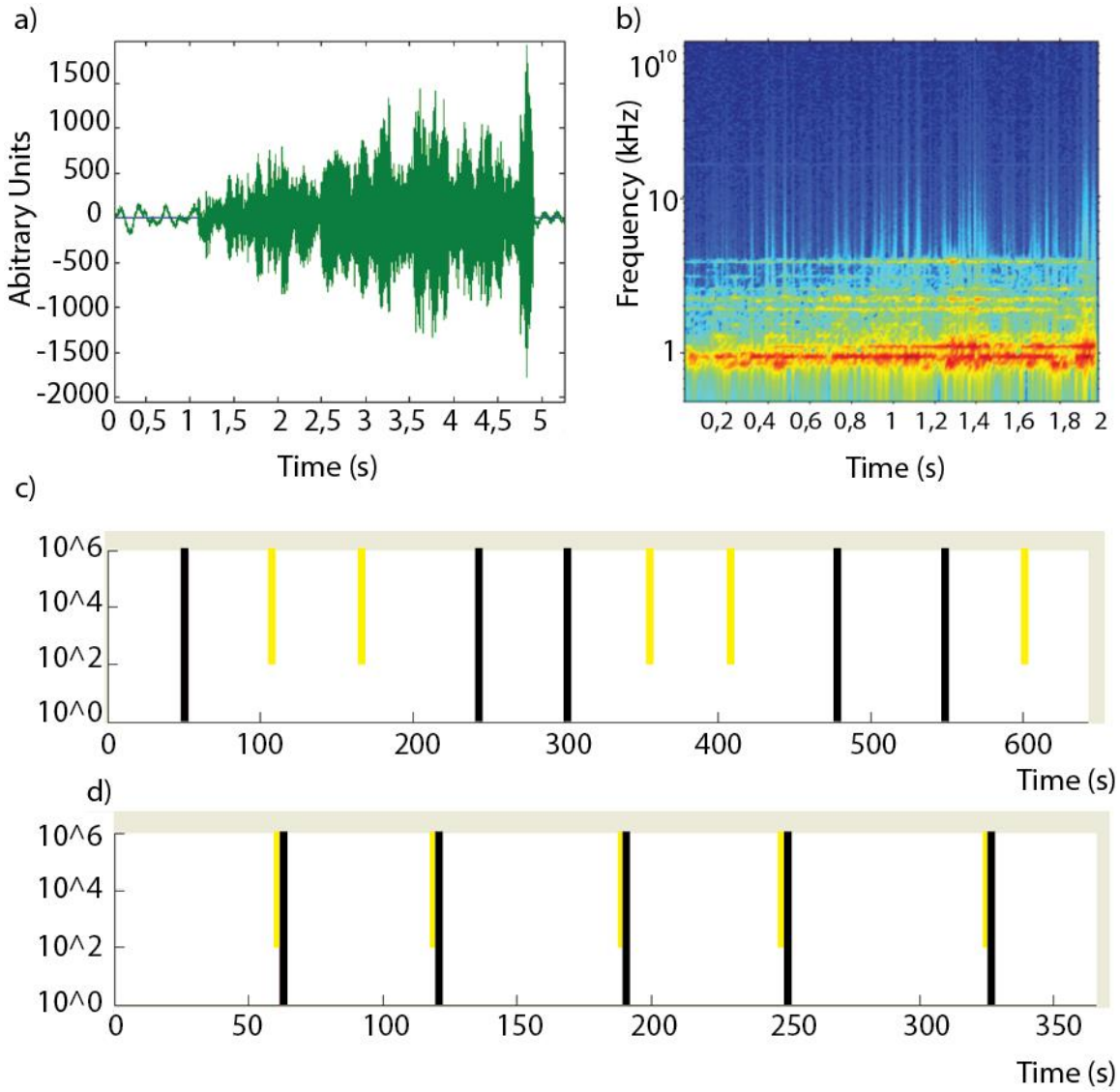


Fig 2) Sound cue and conditioning protocols

a) Waveform representation of the sound cue used for fear conditioning. b) Spectrogram representation of the sound cue used. c) Protocol for unpaired conditions; the black stripe rising from the bottom up, displays the onset of a shock with 0,7mV, whereas the yellow stripe from the top illustrates the activation of the complex sound d) Protocol for paired conditions; mice passing this protocol received after 60 seconds the first sound (yellow stripe), followed immediately by a shock (black stripe). After this first event mice had a resting period of at least 50 seconds to maximal 75 seconds until they received the next paired stimulus.

Results

Establishment of a CRTC1 immunohistochemical staining

Previous studies showed the existence of CRTC1 and its neuronal nuclear translocation activity in primary neuronal cell cultures due to stimulation with KCl or treatment with the $GABA_A$ receptor antagonist bicuculline (Zhou et al., 2006; Kovács et al., 2009; Li et al., 2009; Ch'ng et al., 2012). *In vivo* experiments revealed CRTC1 expression in excitatory neurons in the dentate gyrus of the dorsal hippocampus and a link between CRTC1 and memory formation (Sekeres et al., 2012). To observe CRTC1 *in vivo* in the auditory cortex and reveal its translocation, I established an immunohistochemistry approach (Fig 3). Therefore, I took four wildtype *C57Bl/6J* (Material and Methods; Animals) anesthetized the animals with ketamine (Material and Methods; Solutions and Antibodies), perfused them (Material and Methods; Perfusion) and removed their brain. I cut the PFA fixed brains into 70µm thick sections with a vibratome to gain access to the auditory cortex (Material and Methods; Vibratome). Subsequently, I stained sections comprising the ACx with DAPI, a substance intercalating into the DNA and therefore marking cell nuclei and a fluorescent CRTC1 antibody (Material and Methods; DAPI and CRTC1 immunohistochemistry staining) and imaged slices with an inverted microscope (Material and Methods; Confocal microscopy) (Fig 3).

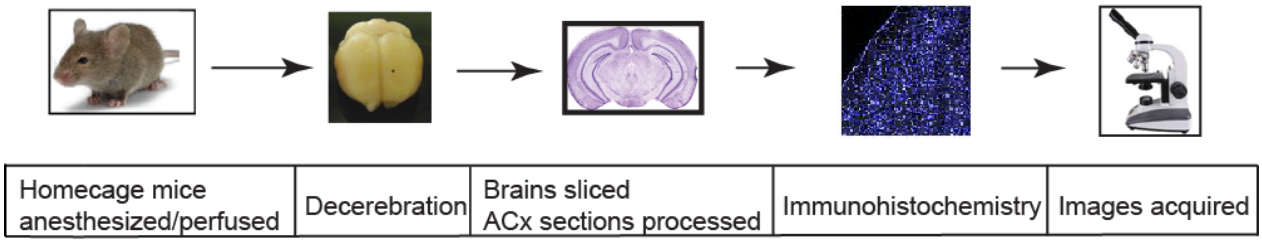


Fig 3) Working process to acquire information about CRTC1 in naïve mice in the ACx

Mice were anesthetized with ketamine, perfused, decapitated and the brains were removed. Brains were cut with a vibratome in a free floating manner and immunohistochemical marked with DAPI and CRTC1.Images were taken with an inverted confocal microscope.

I tested different dilutions (1:50,1:100,1:500,1:1000,1:5000,1:10 000) of several antibodies (TORC1 monoclonal rabbit, Cell signaling technology Inc.#2501S and TORC1 monoclonal rabbit, Cell signaling technology Inc. #2587) to obtain an immunohistochemical staining of CRTC1. DAPI provided a strong signal present in all layers of the visualized primary auditory cortex as well as a

clearly defined CRTC1 signal, in accordance with a previously published paper, using the TORC1 monoclonal rabbit, Cell signaling technology Inc. #2587 antibody,1:5000 diluted (Sakamoto et al., 2013). I could observe spherical structures of the CRTC1 signal around cell nuclei as well as filled circles of CRTC1 expression in the nucleus (Fig 4a top right close-ups 1, 2, 3). To test the specificity of the DAPI/CRTC1 staining, I treated control slices with the primary antibody against CRTC1 but without a secondary antibody, necessary to visualize the proper binding of the 1st antibody. Beside that I made another control staining using only the secondary antibody without the addition of the first antibody which should lead to no specific interaction due to the lack of the unique 1st antibody, recognizing intracellular CRTC1. The control stainings (Fig 4b, 4c) showed no concurrence with the generated DAPI/CRTC1 immunohistochemistry staining. I analysed the correlation of the acquired DAPI and CRTC1 signals for the auditory cortex layer 2/3, layer 4 and layer 5/6. Cells were detected by DAPI and the mean intensity of DAPI and CRTC1 signals were calculated for each cell (Fig 4d). A correlation analysis showed a negative correlation between both signals, indicating that particularly bright nuclei are only weakly fluorescent in the CRTC1 channel. Taken together, these results describe the establishment of a functional DAPI/CRTC1 immunohistochemistry staining, supported by analysis data, showing a correlation for the obtained signals of DAPI and CRTC1.

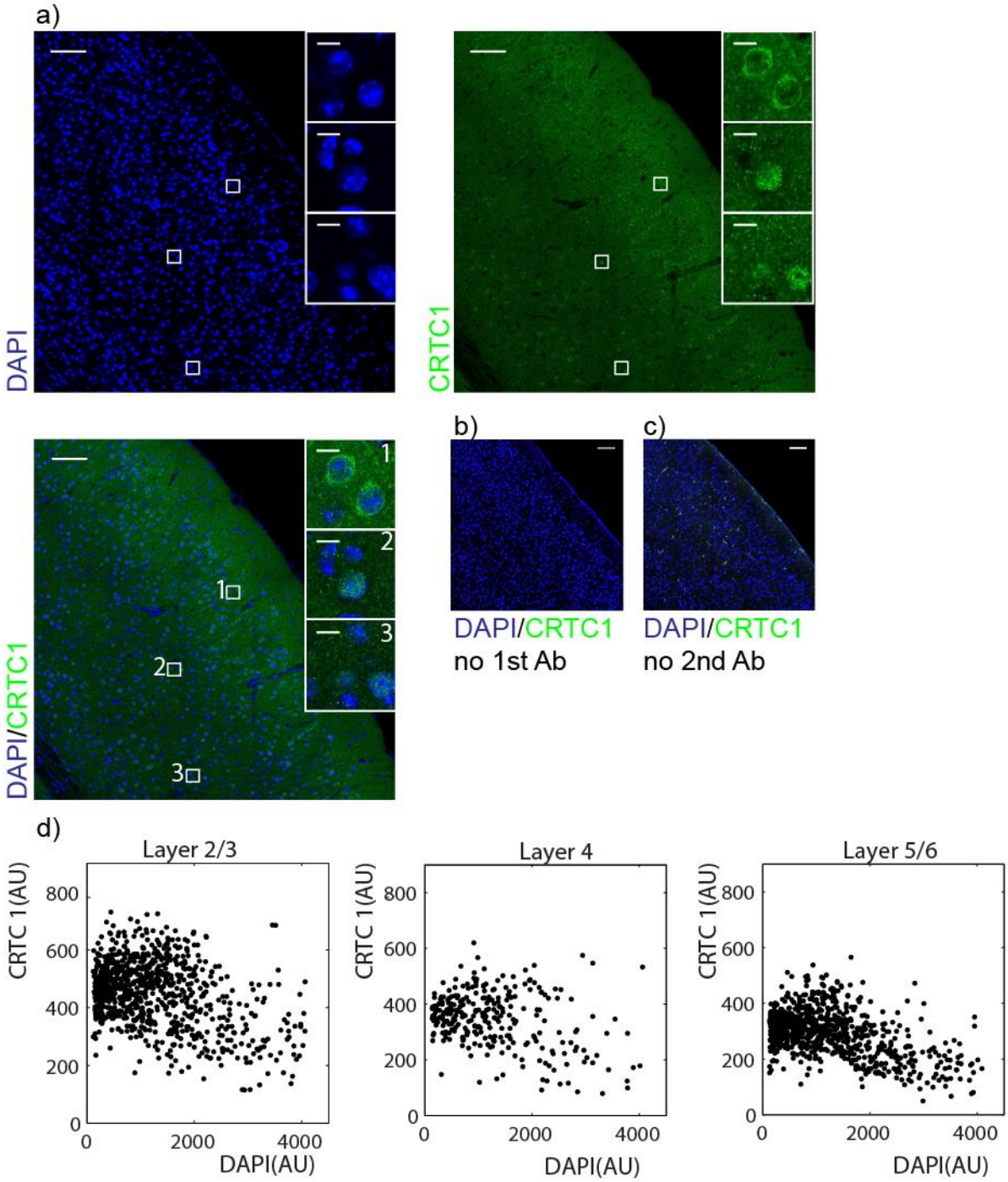


Fig 4) DAPI/CRTC1 immunohistochemistry staining; Single cell analysis for signal coincidence of DAPI and CRTC1

a) DAPI staining of the primary ACx (top left); CRTC1 staining of ACx (top right); merged image of DAPI and CRTC1 signal(bottom left); three chosen close ups show single cells; layer 2/3 illustrates circular structures while layers 4 and 5/6 display rather ball shaped patterns (Scale bar: 100µm, close up: 10µm) b) Control image, slice treated with only 2nd antibody , depicting no CRTC1 expression pattern (Scale bar: 100µm) c) Control image, slice treated with only 1nd , showing no CRTC1 related signal (Scale bar: 100µm) d) Signal intensity correlation of DAPI and CRTC1 illustrates single cell plots showing a correlation of weaker DAPI signal with a rather broad CRTC1 signal in layer 2/3 and 4. The signals in layer 5/6 depict a correlation between fainter DAPI and CRTC1 signals. Layer 2/3, r=-0.4687; p<0,001; n=935; Layer 4 r=-0.38052; p<0,001, n=313; Layer 5/6, r=-0.54956; p<0,001; n=1060

Establishment of a NeuN immunohistochemical staining

The previous analysis showed that CRTC1 is only expressed in a subset of cells within the ACx. To test if these cells are neurons I established a staining with the nuclear marker DAPI, to localize nuclei, and the antibody anti-NeuN which is a neuronal specific marker, able to identify post-mitotic neurons (Material and Methods; DAPI and NeuN staining). I used brain slices of 4 naïve mice, obtained from performing the same protocol "Establishing of CRTC1 immunohistochemistry staining" with 70µm thickness of the slices and observed a DAPI signal as well as a NeuN signal in 20µm depth of the slices (Fig 5a). To ensure the specificity of the acquired images I made control experiments, adding only the 1st anti-NeuN antibody or only the 2nd 633 goat anti guinea pig antibody (Materials and Methods, Solutions and antibodies). I detected no significant NeuN intensity signals while providing only one of the two necessary antibodies (Fig 5b). Looking closer into the established images, a positive correlation of the two signals could be observed (Fig 5a close-up arrow 2), implying that the visualized cells are neurons. Besides that, also divergences of the signals were recognized, unmasking some cells as non-neuronal populations (Fig 5a close-up arrow 1). Up to 766 cells in layer 2/3, 281 cells in layer 4 and 868 cells in layer 5/6 got registered by their expressed signal intensity (Fig 5d). After comparing the DAPI and NeuN signal intensities, I observed a concurrence of the weaker DAPI signals with a rather broad signal of NeuN in all layers for a majority of spotted cells. Taken together, this experiment shows the labelling of a subset of neurons in the auditory cortex, uncovered with an anti-NeuN staining for post mitotic neuronal populations in almost all cortical layers. Layer one acts as an exception because it comprises only very few inhibitory neurons (Kubota et al., 2011).

CRTC1 positive cells are neurons

To directly adress the question if CRTC1 positive cells depict a subset of neurons, I established a double immunohistochemistry staining with CRTC1 and NeuN. I used 4 mice and performed the established workflow (Fig 3) to generate free floating brain slices of mice. I stained these brain slices with anti-NeuN, CRTC1 antibodies and DAPI (Fig 6) (Material and Methods, NeuN and CRTC1 staining).

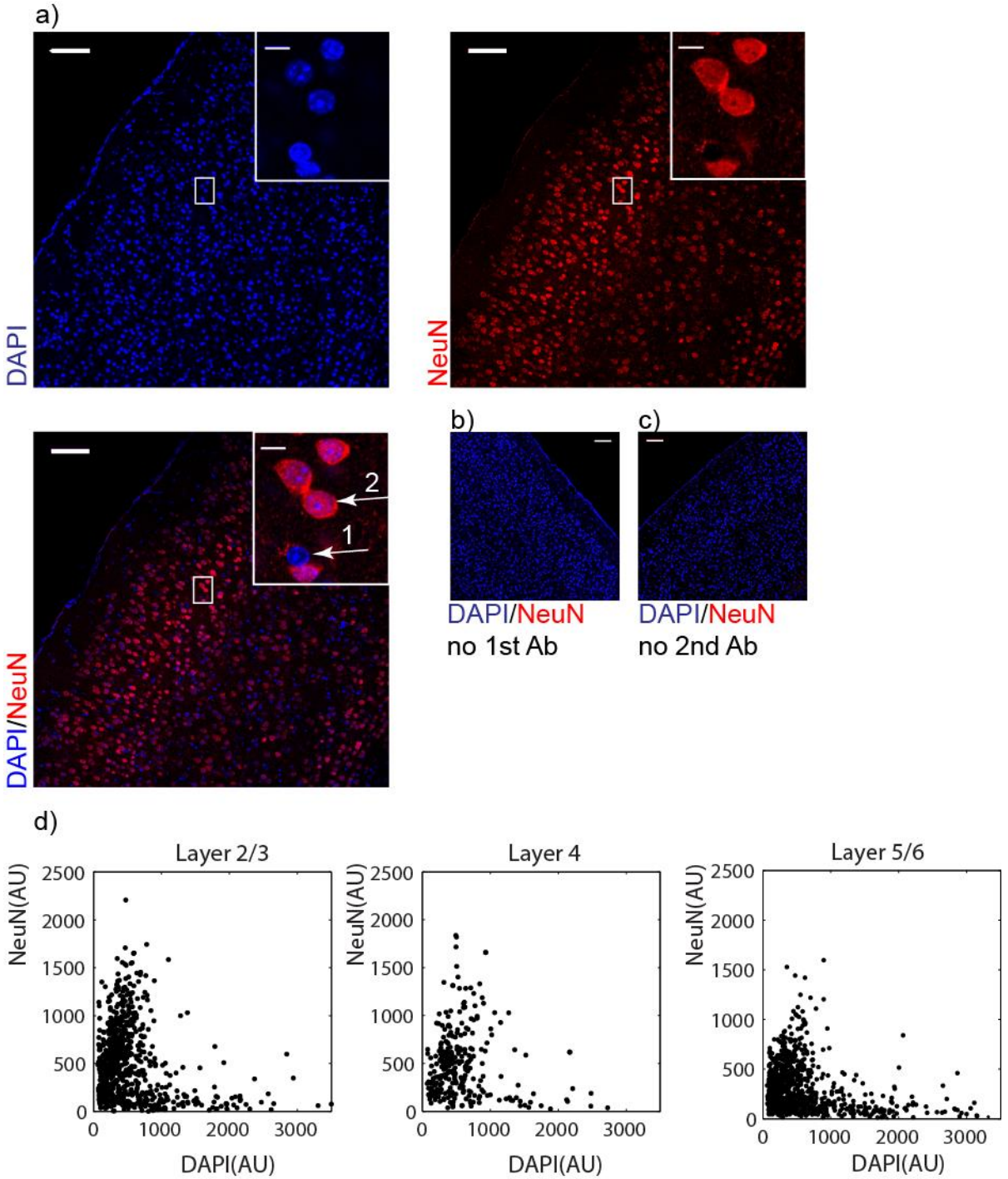
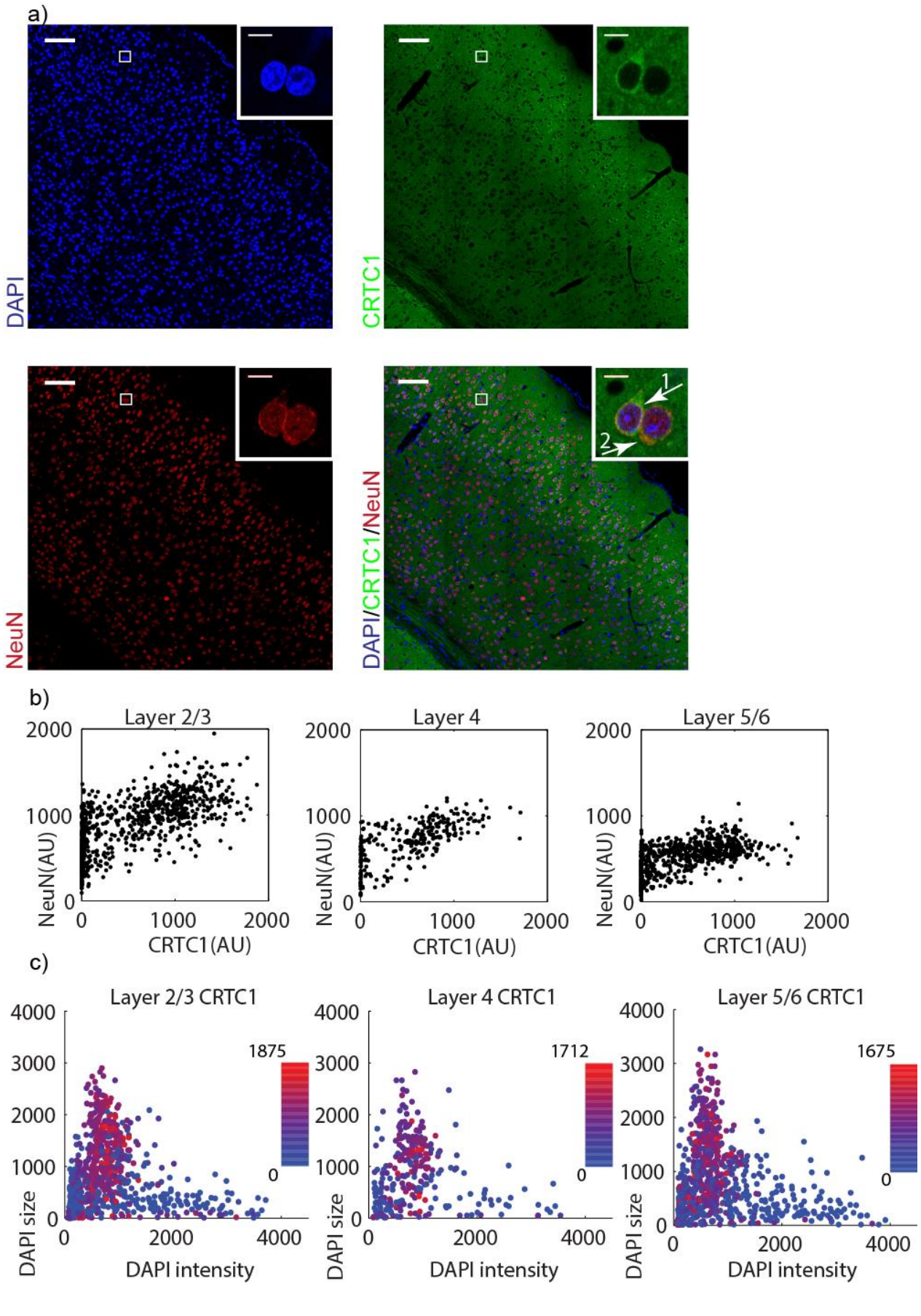


Fig 5) DAPI/NeuN immunohistochemistry staining; Single cell analysis for signal coincidence of DAPI and anti-NeuN

a) DAPI staining of the primary ACx (top left); anti-NeuN staining of ACx (top right); merged image of DAPI and anti-NeuN signals (bottom left); three chosen close ups show single cells; Arrow 1 displays a cell with DAPI but no NeuN signal; Arrow 2 displays a cell with DAPI and NeuN signal implying this cell as a neuron (Scale bar: 100µm, close up: 10µm) b) Control image, slice treated with only 2nd antibody (Scale bar: 100µm) c) Control image, slice treated with only 1nd (Scale bar: 100µm) d) Signal intensity correlation of DAPI and NeuN, illustrates single cell plots showing a correlation of weaker DAPI signals with the broad signal of NeuN in all layers. Layer 2/3, r=-0.19378; p<0,001; n=766; Layer 4, r=-0.10733; p<0,001; n=281; Layer 5/6, r=-0.22709; p<0,001; n=868

DAPI visualizes all existing cells in the ACx due to its common incorporation into DNA (Fig 6a top left). The staining for the CRTC1 expression in the same slice depicted ring like structures around the nucleus, while NeuN depicts again a completely filled nucleus of marked cells (Fig 6a top right, bottom left). When looking closer into merged images (Fig 6a bottom right), one can observe CRTC1 and NeuN positive cells (Fig 6a close-up arrow 1) as well as only NeuN positive cells (Fig 6a close-up arrow 2). By plotting the NeuN and CRTC1 signals for all the layers of the auditory cortex against each other, it depicts neurons being positive for NeuN but negative for the CRTC1 signal, indicated by the signal cluster on the NeuN axis (Fig 6b). Especially layer 2/3 shows many NeuN positive cells with a broad variety in signal intensity but no correlation with the CRTC1 expression signal (Fig 6b left graph). Nonetheless, in all layers a positive correlation between the NeuN and CRTC1 signals was observed, indicating CTRC1 positive cells seem to be part of a neuronal subpopulation (Fig 6b). I plotted the nuclear size of all detected cells (Fig 6c and d,), of layer 2/3, layer 4 and layer 5/6 and compare them to the intensity of the DAPI signal as well as to the intensity of the CRTC1 expression levels or the intensity of the NeuN signals (Fig 6c and d). This approach allowed me to filter out neurons, due to their expression of NeuN and thereby excluding supporting glia cells. Besides that also neurons exhibiting non-evaluable properties due to the imaging settings were excluded. Therefore, cells got recognized based on their respective DAPI fluorescence signal and plotted to their corresponding nuclear size (Fig 6c, Fig 6d). Each dot represents a single cell and its respective colour shows the intensity of the CRTC1 or NeuN signal according to the colour code. The intensity levels are adjusted for each plot and can range from e.g. 0 to 1875 (Fig 6c Layer 2/3 CRTC1). We chose a fictive 2D area with the lowest value for the nuclear size being 1500, and a signal intensity range of DAPI from 200 to 2000 arbitrary units. This combined settings represents the highest CRTC1/NeuN signal to be the chosen zone for further analysis. Taken together these results showed that CTRC1 positive cells are a part of a neuronal subpopulation, indicated by a CRTC1 and NeuN signal correlation on the basis of single cells.


a)
DAPI
CRTC1
NeuN
DAPI/CRTC1/NeuN
1
2
b)
Layer 2/3
Layer 4
Layer 5/6
NeuN(AU)
CRTC1(AU)
0
1000
2000
c)
Layer 2/3 CRTC1
Layer 4 CRTC1
Layer 5/6 CRTC1
1875
1712
1675
DAPI size
DAPI intensity
3000
4000

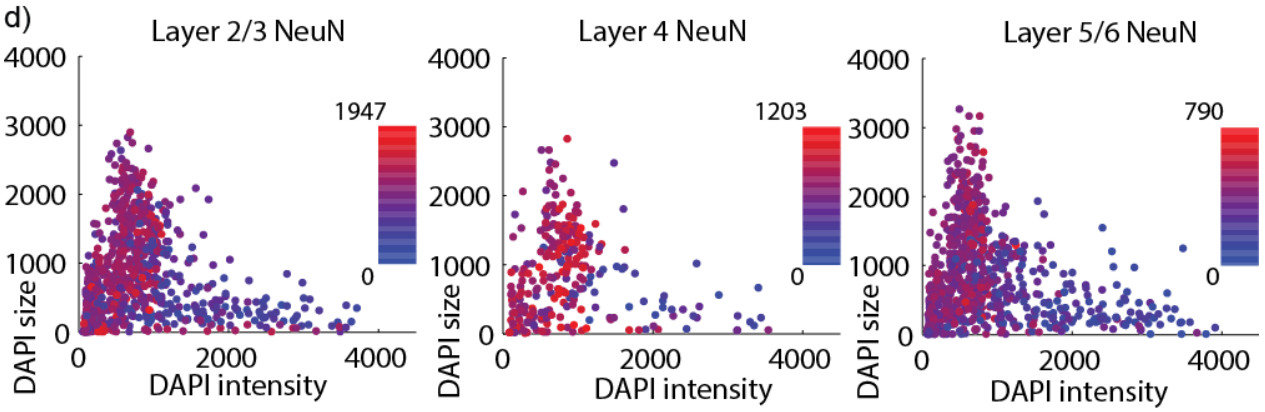


Fig 6) DAPI/NeuN/CRTC1 immunohistochemistry staining; Single cell analysis for signal coincidence of NeuN and CRTC1; Expression level of NeuN or CRTC1 dependent on the size and brightness of signal of the calculated cells

a) DAPI staining of the primary ACx (top left); CRTC1 staining of ACx (top right); NeuN staining of the ACx (bottom left); merged image of the same slice treated with DAPI, NeuN and CRTC1 (bottom right); chosen close ups show single cells; arrow 1 displays a cell with DAPI, NeuN and a CRTC1 expression signal; Arrow 2 displays a cell only with DAPI and NeuN (Scale bar: 100µm, close up: 10µm) b) Correlation plot between CRTC1 and NeuN signals; roughly 1/3 of the 796 visualized cell in layer 2/3 show a NeuN signal only; the majority of cells in all the layers depict a correlation of both signals for single cells ; Layer 2/3, r=0.6595; p<0,001; n=796; Layer 4, r=0.66688; p<0,001; n=271; Layer 5/6, r=0.56043; p<0,001; n=790 c) Intensity level of CRTC1, plotted against the DAPI intensity signal of recognized cells and the size of detected cells; Layer 2/3, maximum intensity=1875, n=796; Layer 4, maximum intensity=1712, n=271; Layer 5/6, maximum intensity=1675, n=790; d) Intensity level of NeuN, plotted against the DAPI intensity signal of recognized cells and the size of detected cells. Layer 2/3, maximum intensity=1947, n=796; Layer 4, maximum intensity= 1203, n=271; Layer 5/6, maximum intensity =790, n=1140;

Establishment of a CaMKII staining in the auditory cortex to label excitatory neurons

Previous in-vitro studies, focusing on the expression of CRTC1 in different cell types could show a presence of CRTC1 in inhibitory neurons as well as in excitatory neurons. After stimulation of hippocampal neurons in cell culture with bicuculline, translocation of CRTC1 from the dendrites into the nucleus could only be observed in excitatory neurons (Ch'ng et al., 2012). Further *in vivo* studies revealed CRTC1 expression only in excitatory neurons in the dentate gyrus but not in glia cells (GFAP) or interneurons (Gad67) (Sekeres et al., 2012). To observe if these previous results hold also true for the auditory cortex, 4 naïve mice were sacrificed and the same protocol as for the establishment of the DAPI-CRTC1 staining (Fig 3) was performed. Brain slices were stained with DAPI and the CaMK II antibody (Material and Methods, Solutions and Antibodies). Subsequently they were imaged (Fig 6a) and analysed.

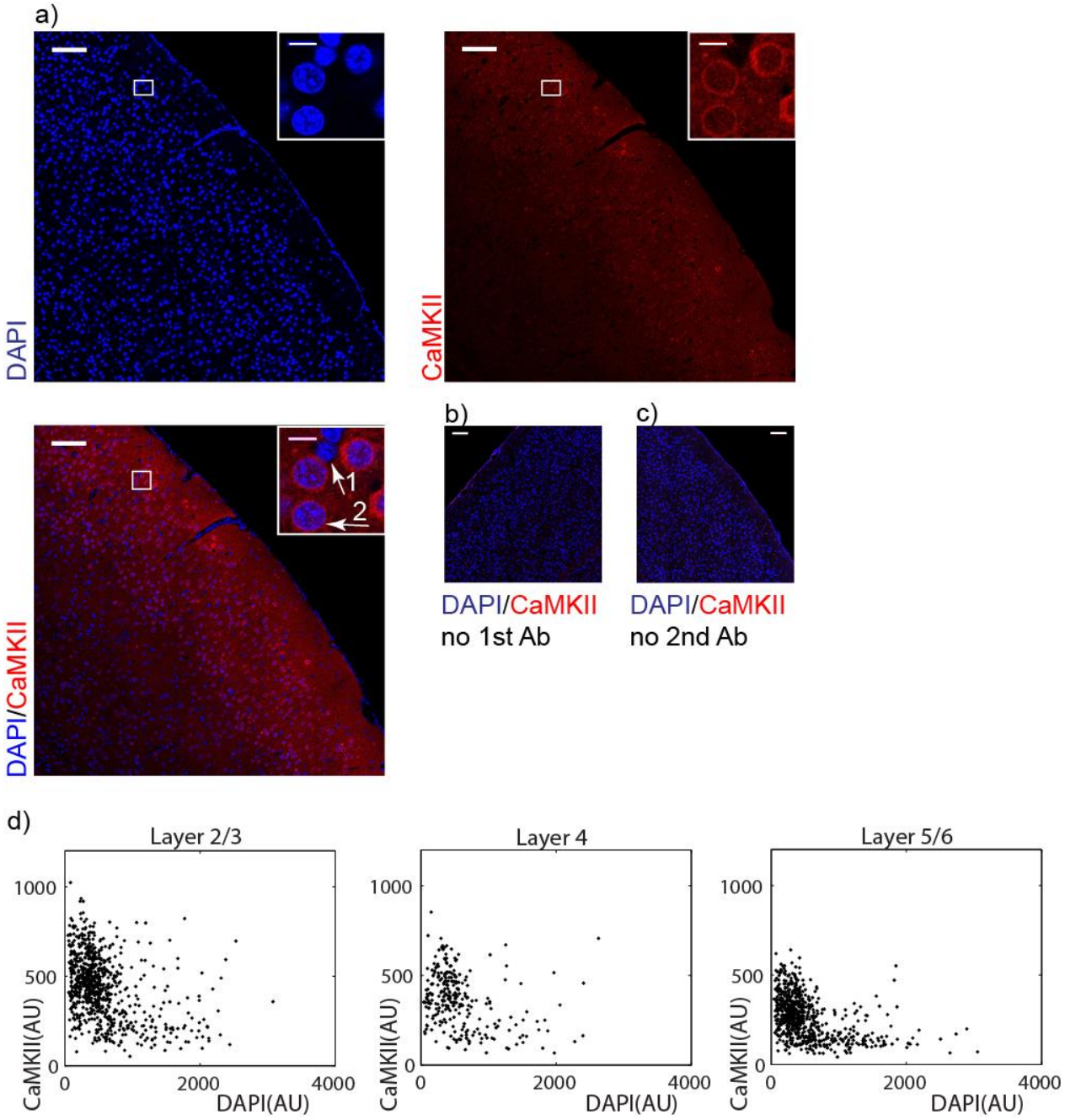


Fig 7) DAPI/CaMKII immunohistochemistry staining; Single cell analysis for signal coincidence of DAPI and CaMKII

a) DAPI staining of the primary ACx (top left); CaMKII staining of ACx (top right); merged image of DAPI and CamKII signal (bottom left); three chosen close ups show single cells; arrow 1 displays a cell with DAPI but no CaMKII signal; arrow 2 displays a cell with DAPI and CaMII signal implying that this cell is an excitatory neuron (Scale bar: 100µm, close up: 10µm) b) Control image, slice treated with only 2nd antibody (Scale bar: 100µm) c) Control image, slice treated with only 1nd (Scale bar: 100µm) d) Signal intensity correlation of DAPI and CaMKII, illustrated single cell plots, show the correlation of weaker DAPI signals with a rather broad signal of CaMKII in all layers Layer 2/3, r=-0.38911; p<0,001; n=768; Layer 4, r=-0.30653; p<0,001; n=272; Layer 5/6, r=-0.43155; p<0,001; n=694

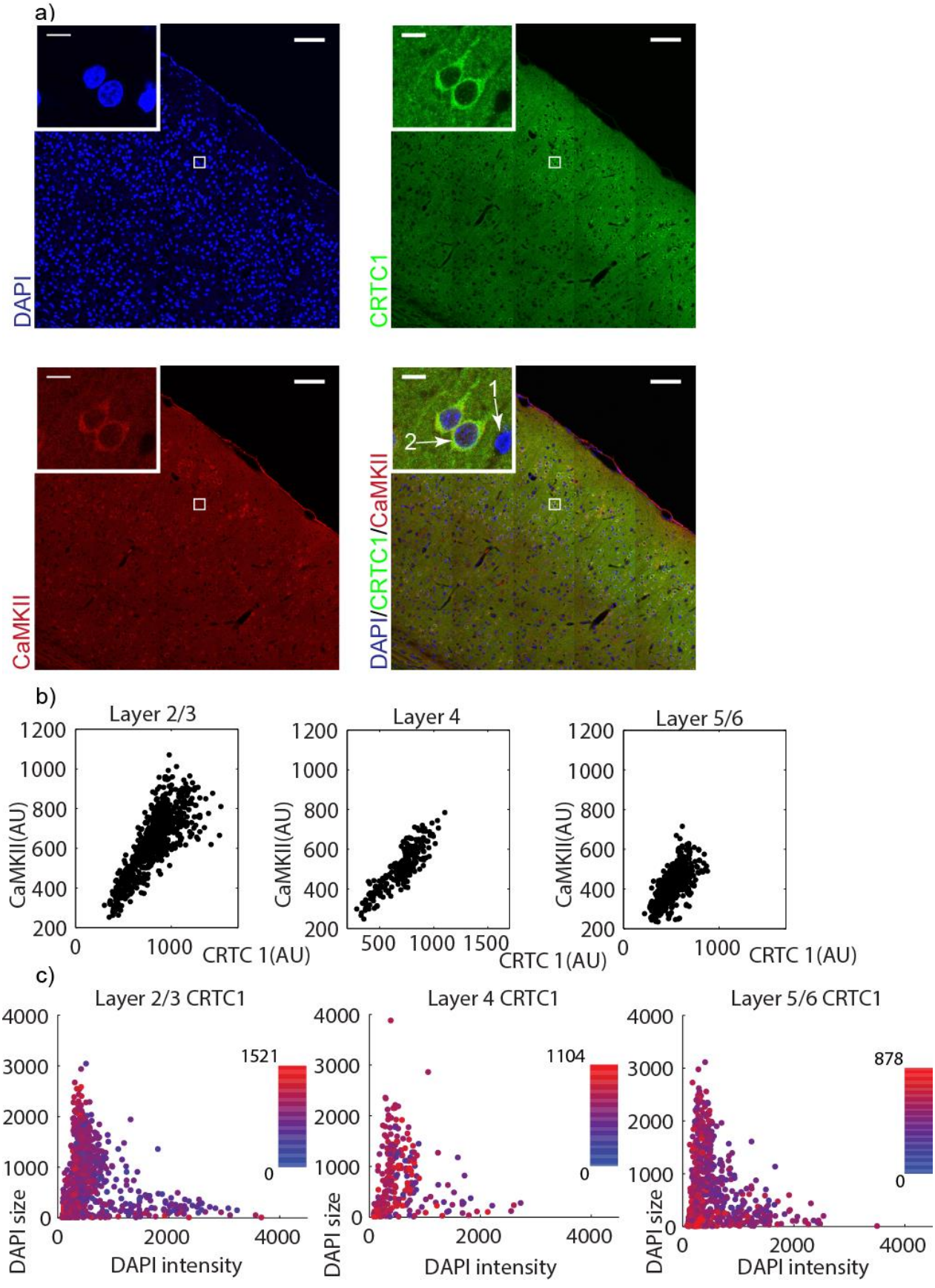
With the addition of DAPI to the slices I visualized nuclei of all different cell types in the auditory cortex (Fig 7a top left). I observed cytoplasmic expression of CaMKII in excitatory neurons, indicated by spherical structures around detected nuclei (Fig 7a top right). Looking closer into the acquired images I observed that many cells exhibit the presence of CaMKII in their cytoplasm in all layers (Fig 7a bottom left close-up, arrow 2, example for layer 2/3). Beside those excitatory neurons, I found also smaller cells with no CaMKII expression, suggesting that these cells are glia cells (Fig 7a bottom left close-up, arrow 1). To ensure the specificity of the acquired images I performed control experiments, adding only the 1st CaMKII antibody (Fig 7b) or only the 2nd 633 goat anti mouse antibody (Fig 7c) (Materials and Methods, Solutions and antibodies). I ascertained no significant CaMKII intensity signals while providing only one of the two necessary antibodies. When opposing the DAPI and the CaMKII signal intensities for individual cells, I observed a concurrence of the weaker DAPI signals with a rather broad signal of CaMKII in all layers, leading to the validation that excitatory neurons are indeed in the auditory cortex. Cells with excessively bright DAPI signals seem not to represent a neuronal population (Fig 7d). Taken these results together I showed the establishment of a specific staining, recognizing excitatory neurons and distinguish them from other cell types.

CRTC1-expressing cells in the auditory cortex are excitatory neurons

With the established CaMKII staining, labelling excitatory neurons, I next wanted to answer the question if neurons, expressing CRTC1, are excitatory neurons. Therefore, I combined two established staining protocols and generated a co-staining for CRTC1 and CamKII. Four naïve mice were used for this approach. Brain slices of the ACx were generated, according to the previously established protocol (depicted in Fig 2). I observed the same expression patterns for the CRTC1 as well as for the CaMKII signal as in the previously produced staining's.

Both signals showed similar orbital structures around the nucleus, focusing on layer 2/3 of the auditory cortex (Fig 8a top right, bottom left). By comparing the signals of CRTC1 and CaMKII, a positive correlation for all examined layers was observed, leading to the assumption that a majority of CRTC1 positive cells are excitatory neurons. The analysis for layer 2/3 depicted a broad distribution of single cells but also a significant positive correlation for the compared signal intensities (Fig 8b left graph). A congruence for both signals was observed in layer 4. When compared to layer 2/3, it depicts cells with lower signal but less signal intensity spread. Layer 5/6 exhibits also lower signal intensity levels for CRTC1 and CaMKII but a very tight cluster of correlating signals compared to the other layers (Fig 8b).

When plotting the signal intensity of CRTC1 or CamKII against the size and the DAPI signal, it showed a clear match of CRTC1 and CaMKII signals towards cells with a lower DAPI intensity. Besides that signal coincidences could also be observed for a broad band of cells depicting differences in their size. If only one of the two signal intensities were investigated, a congruence between the displayed patterns could be detected, leading to the conclusion of similar expression levels for CRTC1 and CaMKII in mostly all of the analysed cells (Fig 8c and Fig 8d). Taken together, with these results I showed that CRTC1 expressing cells are mostly excitatory neurons based on the coincidence signal detection of CaMKII and CRTC1 in single visualized cells.


a)
DAPI
CRTC1
CaMKII
DAPI/CRTC1/CaMKII
1
2
b)
Layer 2/3
Layer 4
Layer 5/6
CaMKII(AU)
CRTC 1(AU)
c)
Layer 2/3 CRTC1
Layer 4 CRTC1
Layer 5/6 CRTC1
DAPI size
DAPI intensity
1521
1104
878

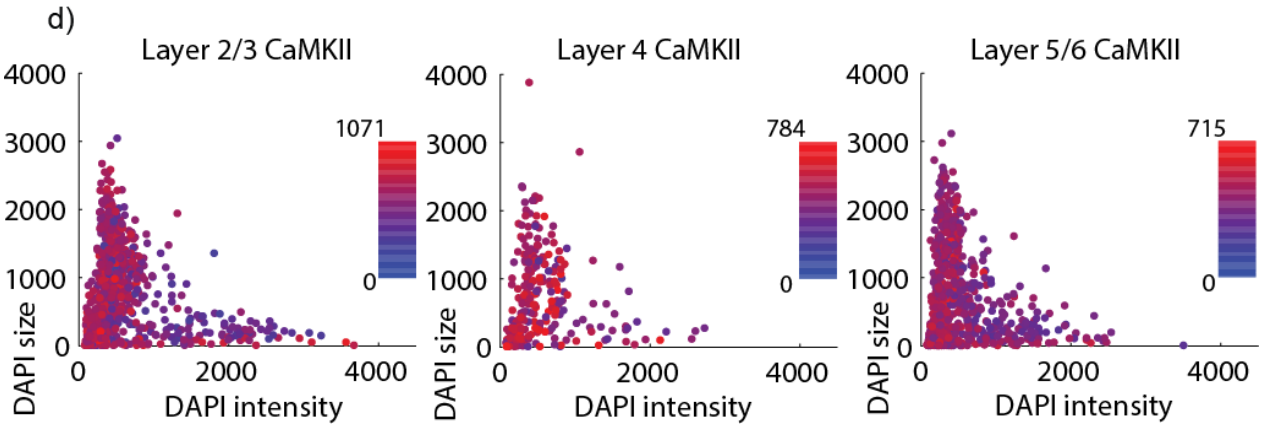


Fig 8) DAPI/CamKII/CRTC1 immunohistochemistry staining; Single cell analysis for signal coincidence of CaMKII and CRTC1; Expression levels of CaMKII and CRTC1 show strongest signals for weaker DAPI signals but over a broader range of cell volume.

a) DAPI staining of the primary ACx (top left); CRTC1 staining of ACx (top right); CaMKII staining of the ACx(bottom left); merged image (bottom right);related close-ups show single cells.; arrow 1 displays a cell only with DAPI indicating a non-excitatory neuron; arrow 2 displays a cell with DAPI/CRTC1/CaMKII labelling, implying that this cell is an excitatory neuron (Scale bar: 100µm, close up: 10µm) b) Correlation plot between CRTC1 and CaMKII signals; Layer 2/3, r=0.7982; p<0,001; n=808; Layer 4, r=0.87387; p<0,001; n=242; Layer 5/6, r=0.68658; p<0,001; n=649; c) Intensity level of CRTC1, plotted against the DAPI intensity signal of recognized cells and the size of detected cells; intensity signal reaches from 1 to 2000; Layer 2/3 , maximum intensity=1521, n=808; Layer 4 , maximum intensity= 1104, n=242; Layer 5/6 , maximum intensity =878, n=649; d) Intensity level of CaMKII, plotted against the DAPI intensity signal and the size of detected cells; Layer 2/3 , maximum intensity =1071, n=808; Layer 4 , maximum intensity =784, n=242; Layer 5/6 , maximum intensity=715, n=649

Establishing a fear conditioning protocol and evaluation analysis to observe the translocation of CRTC1 *in vivo*

After determining CRTC1 positive cells are predominantly excitatory neurons, I wanted to test if behavioural experiences affect the localization of CRTC1. To approach this question I established an associative and a non-associative fear conditioning protocol (Material and Methods, Fear conditioning protocol). The unpaired, non- associative protocol differed from the associative protocol in the presentation of the input signals doing it not in an associative but rather a randomly distributed way (Material and Methods, Fear conditioning protocol). 36 animals were split up into 3 groups of 12 mice each. Mice in group 1 were habituated to the artificial environment and furthermore received a paired fear conditioning protocol. Mice in group 2 were also habituated to the behaviour chamber but obtained a protocol with a random distribution of shocks and tones. Group 3 represented naïve mice which were left in their home-cages for 5 days and only habituated to an anaesthesia procedure. They received neither environment habituation nor conditioning to outer influences.

After 4 days of habituation, mice from group 1 and 2 received their respective associative or non-associative protocols. They were immediately placed back in their home cages after this session. After one hour, mice of all groups got anesthetized, perfused and decerebrated similar to the previously established working protocol (Fig 3). The time point of one hour, to observe CRTC1 translocation after an associative learning paradigm or undergoing a non-associative experience, is based on *in vitro* primary cell culture findings in which after treatment with KCl, CRTC1 nuclear localization showed a main peak after 60 minutes (Zhang et al., 2009).

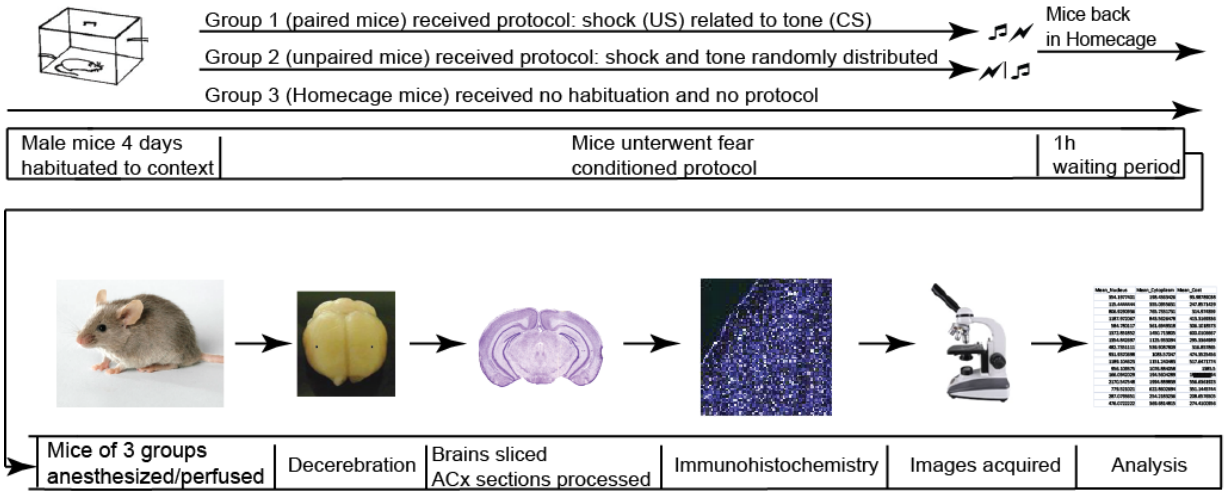
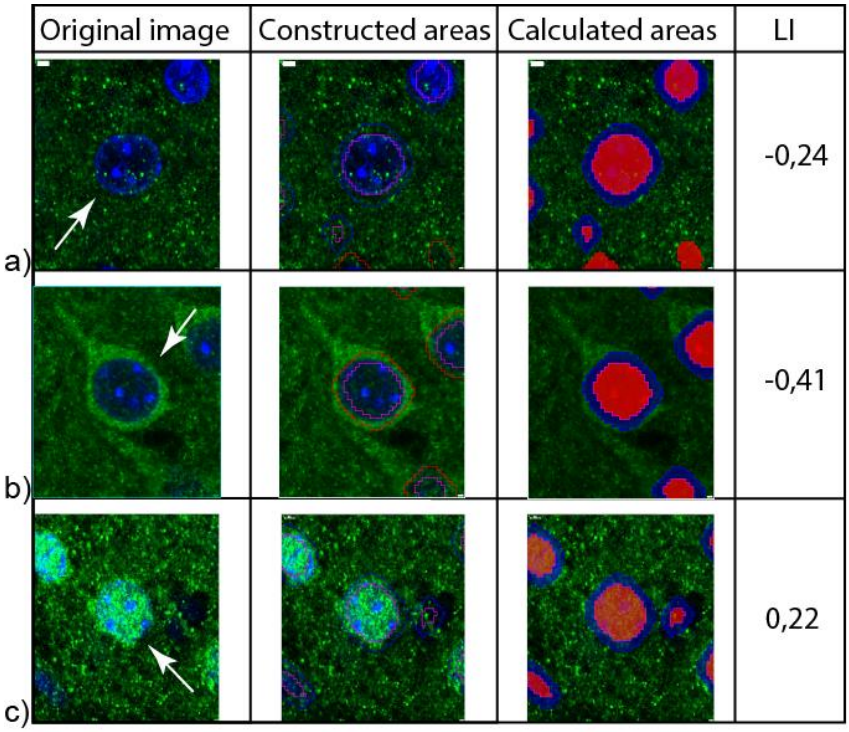


Fig 9) Workflow

Animals were separated into 3 groups of 12; Group1 and 2 were habituated to the artificial environment and further fear conditioned. Group 1 received an associative fear learning protocol while group 2 underwent a non-associative protocol. Group 3 were naive mice, receiving no habituation/ exposure to a protocol. Mice of group 1 and 2 were directly after their fear conditioning sessions placed back into the home cages for 1 h. Afterwards all mice were anesthetized with Ketamine further perfused, decapitated and the brains were removed. Brains were cut with a vibratome in a free floating manner and immunohistochemical marked with DAPI and CRTC1. Images were taken with an inverted confocal microscope and visualized signals evaluated.

Two times two slices per animal, representing the left and right hemisphere of the ACx were imaged. I selected slices illustrating an anterior and a more posterior structure of the primary auditory cortex. The immunohistochemically treated slices (Material and Methods; DAPI and CRTC1 staining) were further analysed (Fig 10). Single cells were detected based on their DAPI signal and included into the analyses (Material and Methods, Analysis of single cells). ROIs were delineated around each detected nucleus and each respective cytoplasm producing two spheres, in each of which the signal intensity of CRCT1 was captured. The average signal intensity of pixels was calculated by subtracting the cytoplasm from the nuclear signal divided by the sum of both

signals for every cell in all layers (Fig 10; LI). This metric is correlated to the relative localization of CRTC1 in the cytoplasm and the nucleus.



Localization Index (LI)

$$\frac{(\text{Nucleus}-\text{Cytoplasm})}{(\text{Nucleus}+\text{Cytoplasm})}$$

LI=1: pure nuclear signal

LI=-1: pure cytoplasmatic signal

Fig 10) Calculated values for recognized and analysis integrated cells

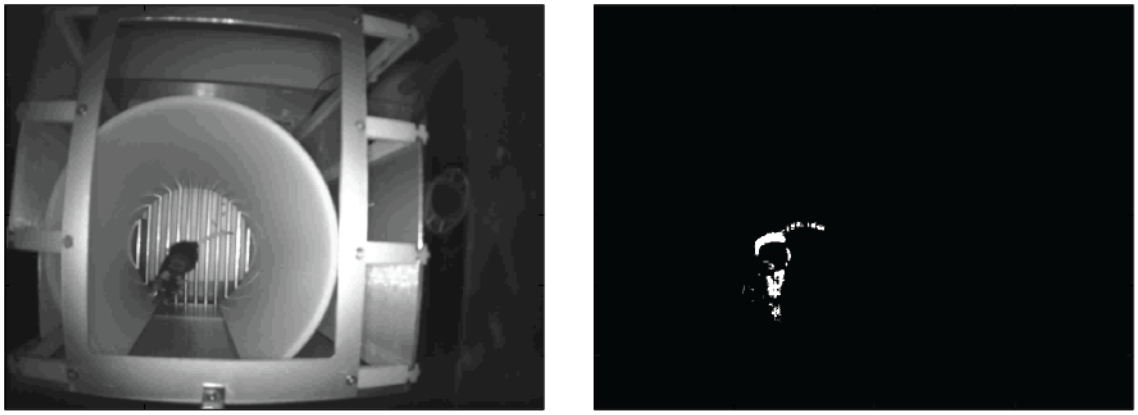
Original images depict the ordinary recording of single cells; Constructed areas show nuclear and cytoplasm recognition via the creation of fictive areas around each of them; Calculated areas illustrate the zones used for the computations of CRTC1 appearance; Values depict single cell localisation of CRTC1

a) blue signal depicts DAPI, green signal represents CTRC1 signal; white arrow marks cell for analysis, fictive areas were drawn around recognized nuclei to generate nuclei and cytoplasm spheres; red represents nuclear- blue cytoplasm areas for calculating the localization of CRTC1 ; Cells shows little till no CRTC1 expression at all, value close to 0 represents no/small amount of CRTC1 in the cytoplasm b) properties similar as before described; analysed cell shows strong signal of CRTC1 in the cytoplasm, represented by its negative calculated value c) depicts a cell with a high expression level of CRTC1 in the nucleus represented by its positive value ; Scale bar for all images 1µm

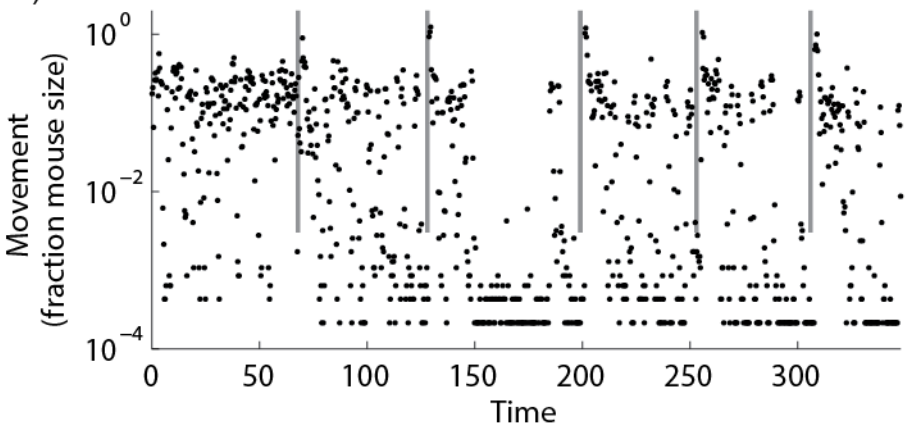
Auditory fear conditioning

To verify that the fear conditioning protocol leads to learning and a memory formation data from eight mice undergoing the protocol were analysed. For that a camera, affixed over the artificial chamber tracked the movements of mice. A difference image analysis calculated significant motion pixels due to locomotion (Fig 11a) (Peter el al., 2012).An increase in freezing after each tone paired with the mild foot shock could was observed (Fig 11b, Fig 11c). This illustrates increasing levels of defensive responses observable in the occurrence of danger. These observations are expected and demonstrate the successful application of the fear conditioning paradigm. It has been shown previously in a large number of studies that such a protocol leads reliably to the formation of a long term association of sound-cue and shock (Peter et al., 2012)

a)



b)



c)

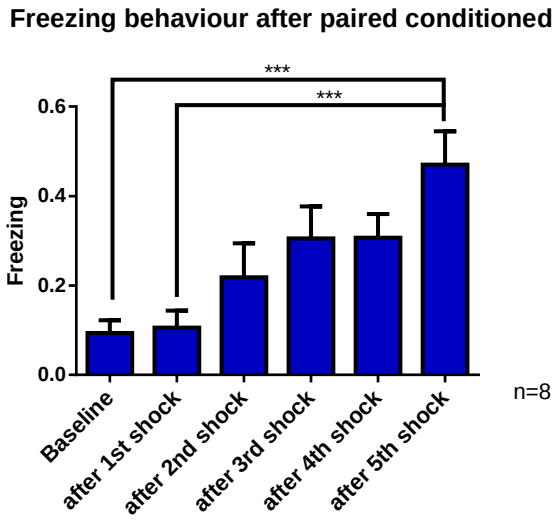


Fig 11) Applied paired fear conditioning protocol induced freezing behaviour

a) Screenshot ("*frame n*") from a mouse placed in the behaviour chamber, receiving a fear conditioning protocol (left); difference image depicting significant motion pixels when movement of the mouse was detected, calculated via a subtraction of a given frame minus the following one; n-(n+1) (right); b) Movement of one performing mouse over time, grey bars depict the shock, single dots illustrate movement of the mouse; The longer the protocol lasted the more freezing was observed c) Freezing duration after the shocks increased over time (Baseline vs. after 5^{th} shock p value<0,001; 5^{th} vs 1^{st} p< 0.001)

Differences in subcellular CRTC1 localization on ACx samples from paired and unpaired conditioned mice

When inspecting the images of the brain slices from various mice from the three behavioural groups, I observed substantial variability in the localization of CRTC1. This was true even in sections obtained from mice within one behavioural group. (Fig. 12). On the one hand this could reflect methodological variability in staining and the estimation of the LI's. On the other hand this could furthermore point towards global differences in the localization of CRTC1 in individual mice reflecting other factors than the behavioural treatment that were not controlled for in the experimental design.

Using the previously established analysis based on single cell values, I merged the values of detected CRTC1 positive cells by averaging to a single value for every mouse. These values were calculated for layer 2/3, layer 4 and layer 5/6 of the auditory cortex (Fig 13 a) b) c) for each of the three behavioural groups. Applying a statistical analysis for data from L2/3, I observed a statistically significant effect of the behavioural treatment on the localization of CRTC1 as reflected by differences in the respective LI's. CRTC1 had a more nuclear localization in naïve (home cage) mice as well as in paired conditioned mice compared to unpaired mice (Fig 13a). (Anova p value <0.025 for all 3 groups, r^2= 0.2056; paired vs. unpaired p< 0.02; Home cage vs. unpaired p< 0.015). Applying the same analysis for L4, I did not detect statistically significant differences in the

LI's, however, a similar trend as in L2/3 towards more nuclear localization in slices from paired and naïve versus unpaired mice was apparent. In layers 5/6 again a statistically significant effect of behaviour on the localization of CRTC1 was observed. Nuclear localization of CRTC1 in naïve (home cage) mice and paired conditioned mice was higher as compared to unpaired mice (Anova p value <0.02, r^2=0.2232; paired vs. unpaired p value <0.015; home cage vs. unpaired p value< 0.025).

In general, independent of the behavioural condition, the LI's were different across layers. Layers 5/6 showed higher LI's as L4, which in turn showed on average higher LI's than L2/3. This could indicate general differences in the localization ratio of CRTC1 in different neocortical layers. Interestingly, it has been shown in the auditory cortex that general activity levels also differ across cortical layers, being highest in supragranular layers (sakata Harris 2008). However, other factors like the general morphology of a cell, which also differs across layers, can also have an impact on the LI.

Taken together, the analysis of CRTC1 localization in differently behaviourally treated groups of mice revealed an effect of behavioural experience, despite substantial inter-individual variability.

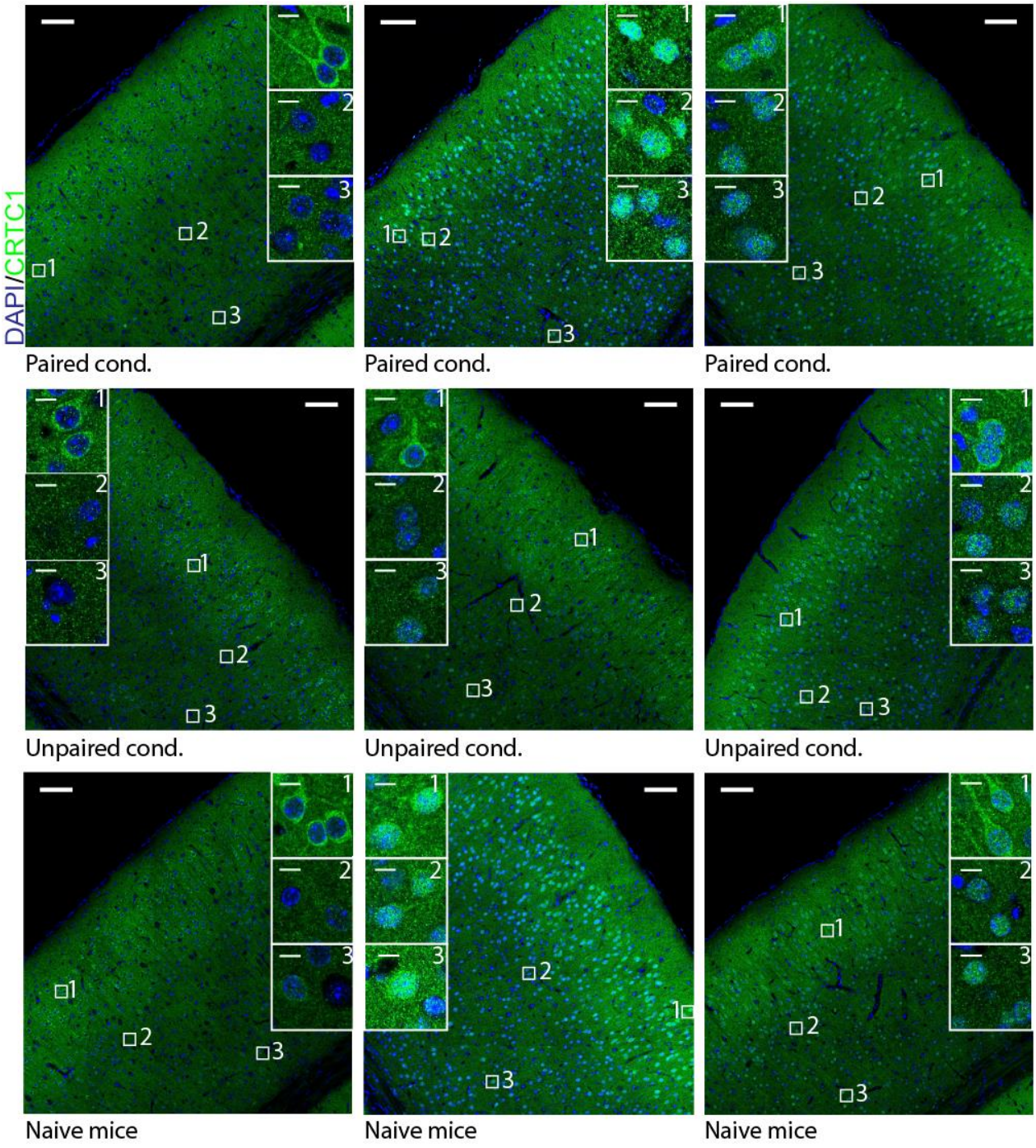
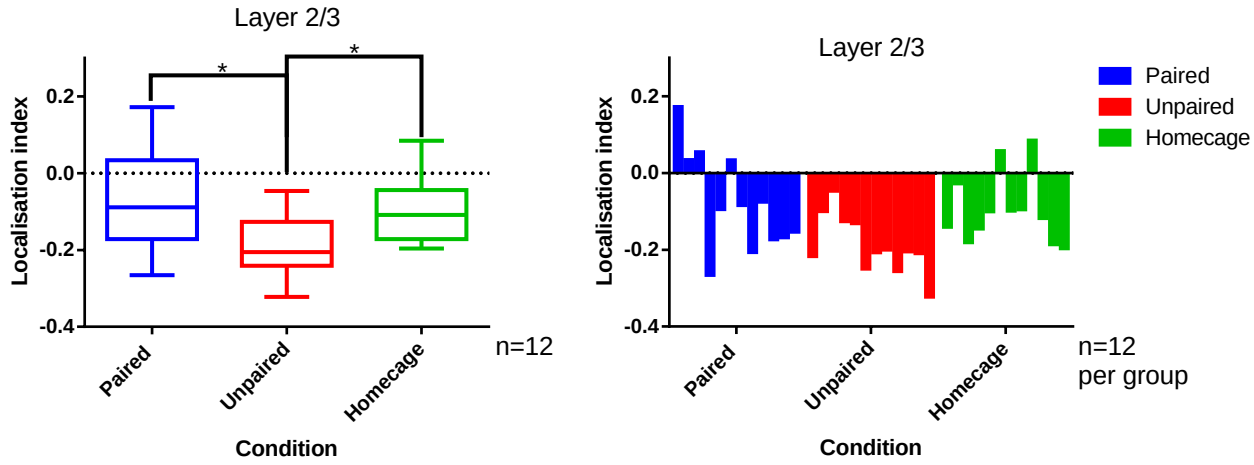


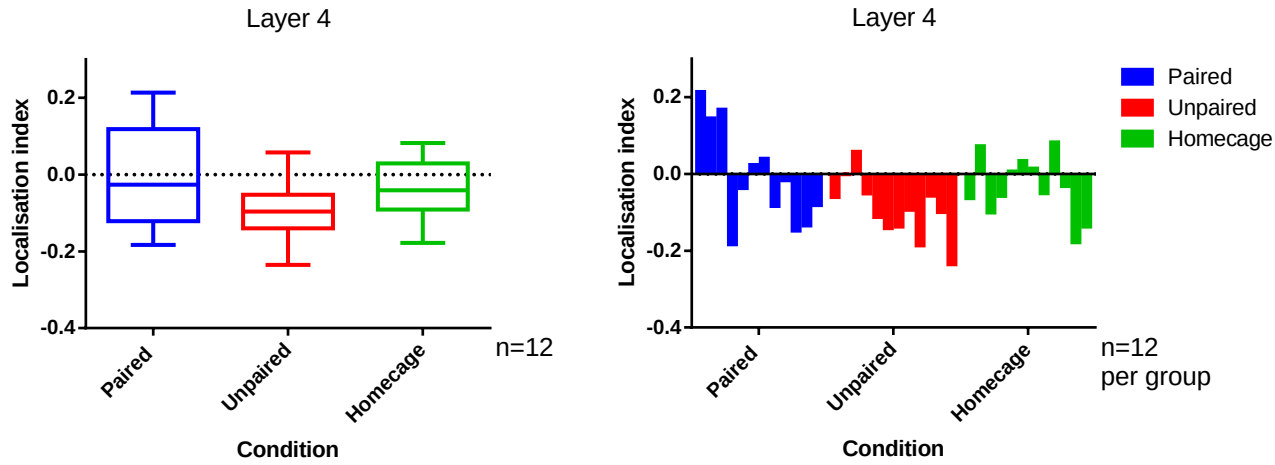
Fig 12) DAPI/CRTC1 immunohistochemical staining for naïve/paired/unpaired mice

3 representative slices, out of 48 acquired slices from each behavioural group are depicted. Close ups in each picture depict neurons of layer 2/3, 4 and 5/6. A trend for each behavioural group can be observed. Paired conditioned as well as naïve mice show predominant CRTC1 occurrence in the nucleus while unpaired mice depict CRTC1 presence particularly in the cytoplasm.

a)


Layer 2/3
Localisation index
0.2
0.0
-0.2
-0.4
*
*
Paired
Unpaired
Homecage
n=12
Condition
Layer 2/3
Paired
Unpaired
Homecage
n=12
per group

b)


Layer 4
Localisation index
0.2
0.0
-0.2
-0.4
Paired
Unpaired
Homecage
n=12
Condition
Layer 4
Paired
Unpaired
Homecage
n=12
per group

c)

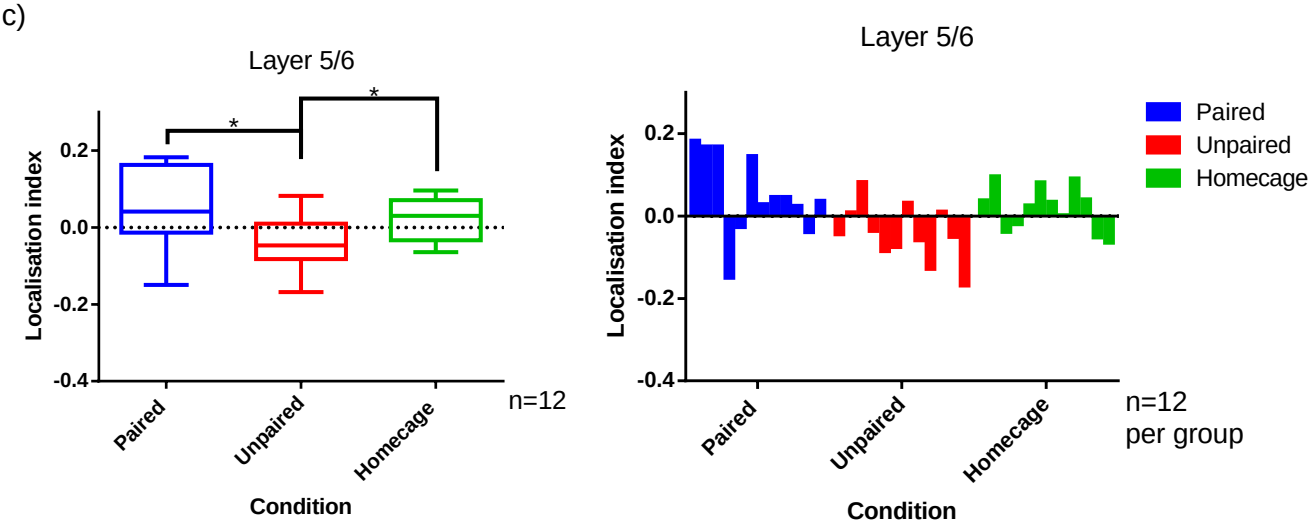

Layer 5/6
Localisation index
0.2
0.0
-0.2
-0.4
*
*
Paired
Unpaired
Homecage
n=12
Condition
Layer 5/6
Paired
Unpaired
Homecage
n=12
per group

Fig 13)

Localization and expression of CRTC1 in layer 2/3, 4 and 5/6 in the auditory cortex

Localization index 1~pure nuclear signal
-1~pure cytoplasmic signal

a) 3 groups of mice receiving different behavioural paradigms were matched to illustrate the localization of CRTC1 in layer 2/3. It shows a significant result between paired and naïve mice to unpaired mice. b) Localization of CRTC1 in layer 4. No significant difference of the localization of CRTC1 was observed c) Paired mice together with naïve mice illustrate a significant difference in the layer 5/6 compared to unpaired mice. Layer 5/6 depicts a common trend of CRTC1 to appear predominantly in the nucleus of neurons compared to layer 2/3 were CRTC1 appears mostly in the cytoplasm; n =12 mice for all groups

Discussion

In my thesis I show that CRTC1 expressing cells in the primary auditory cortex of the mouse are mostly excitatory neurons. I further established a behavioural paradigm and an analysis to observe the translocation properties of CRTC1 in histological sections. I found differences in CRTC1 localization, when comparing mice undergoing learning and memory formation and mice undergoing a non-associative experience. CRTC1 was predominantly located in the nuclei of cells in cortical layer 2/3 and 5/6 in US-CS paired mice compared to unpaired mice, were CRTC1 was rather located in the cytoplasm. No significant differences were observed in CRTC1 localization variation between naïve and paired mice. Independent of the behavioural treatment all groups showed a tendency for higher cytoplasmic signals in layer 2/3, while in layer 5/6 CRTC1 was more detected in the nucleus. With this for the first time direct evidence of experience triggered translocation of CRTC1 was provided.

The contribution of neuromodulators in CRTC1 activity

It has been suggested that the neuromodulators dopamine and norepinephrine can increase cAMP levels, leading to the prolonged presence of CRTC1 in the nucleus and therefore to the induction of CRE dependent gene transcription after a stimulus. Experiments supporting the involvement of dopamine and norepinephrine in memory formation and regulation of CRTC1 revealed increased levels of the neuromodulators after tetanic stimulation as well as an increase during emotional excitement e.g. fearful occurrences (Ch'ng et al., 2012). Looking closer into those specific neuromodulators and their role in fear conditioning, it has been shown that dopamine is essential for cued fear condition and a lack of dopamine impairs stabilization of short term memories (Fadok et al., 2009). The neuromodulator norepinephrine has been suggested to be an important modulator, inducing conditional fear responsiveness, reduced levels of norepinephrine led to an impaired fear response, to modulate acquisition of memory traces and to enhance memory formation (Bremner 1996). It would therefore be interesting to find out if those neuromodulators are the key regulators in the *in-vivo* situation or if there are others involved in the regulation of CRTC1.

Sources of variability in performed experiments

Looking closer in the experiments for my thesis, some interesting sources of variability are found. A source of variability would be the duration of the experiments. Mice receiving their fear conditioning protocol were sacrificed after 1h within a time-window between 10:00am and 13:00pm. Because it has been published that CRTC1 plays an important role in circadian rhythm in the SCN, it could be possible that CRTC1 shows rhythmic properties in the ACx and therefore

a variability in nuclear translocation. Also as described earlier, cell culture and *in-vivo* experiments revealed CRTC1 translocation after 5-15 minutes (Sakamoto et al., 2013 and Li et al., 2009). For my experiments the time point of 60 minutes was chosen to anesthetise and sacrifice mice after conditioning. Due to the lack of information about the neuromodulators acting upstream of CRTC1, it can be possible that the waiting period was set either too short or too long to observe a difference in the translocation and prolonged presence of CRTC1 when comparing paired/unpaired conditioned animals.

Conclusion and outlook

Peter 2012 showed an up regulation of IEGs due to a mild foot shock, independent of an associative or non-associative behavioural paradigm. He showed for the global expression pattern a significant difference between naïve, unpaired and paired mice. CRTC1, in contrast to IEGs, which can distinguish between naïve and paired conditioned mice, seems to make no distinction between naïve and paired mice but shows a significant difference compared to unpaired mice, therefore CRTC1 could be used as a marker for unpaired conditioning due to its specific cytoplasmic presence. My results are interesting in context with previous data shown by Moczulska 2013. She demonstrated an elimination of spines in the auditory cortex after unpaired conditioning but an increase of spines after paired fear conditioning. Considering that CRTC1 is able to signal neuronal activity to the nucleus, it may stimulate spine dynamics via activating CREB1 to induce an enhanced spine elimination.

For the future it would be interesting to find out if only a small fraction of transcription factors (Fig. 14) can depict a precise state of the brain after experiencing a specific event. As described before, the transcription factor CRTC1 demonstrated a significant cytoplasmic occurrence in unpaired mice in the ACx. Are there specific proteins, like transcriptional factors or DNA binding proteins, which represent, due to an increase or decrease of their intracellular concentration a certain state? One step into this direction has been done with focus on the amygdala. There it has been shown that few factors are up regulated after paired conditioning (Lamprecht et al., 2009). Therefore figuring out in more detail which small network of transcription factors can mirror an associative/non-associative condition would be an interesting research direction for the future.

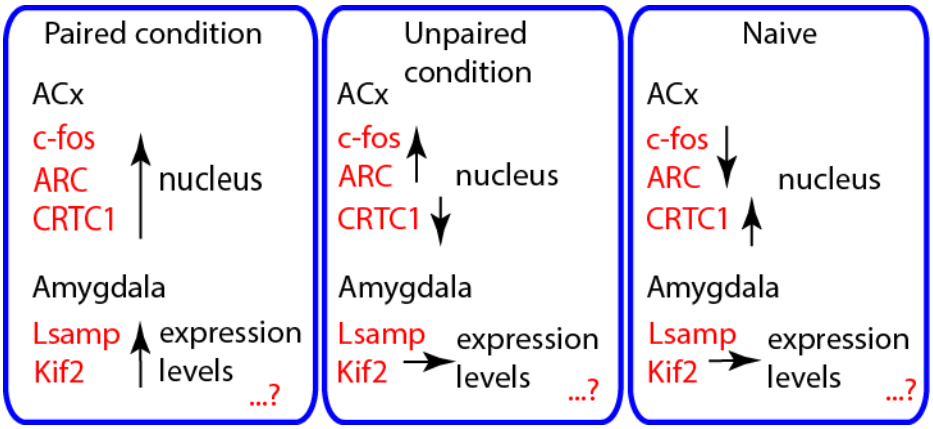


Fig 14) A model of combined transcription factors representing specific behavioural states

The model depicts a small amount of transcription factors such as the immediately early genes c-fos and ARC, which are significantly up regulated in paired/unpaired states compared to a naive state (Peter et al., 2012). CRTC1 translocates into the nucleus in naïve and paired conditions while in unpaired conditioned mice, CRTC1 is predominantly in the cytoplasm. In the amygdala after 5h mRNA expression levels of Lsamp and Kif2 are significant increased in paired conditioned compared to unpaired and naïve mice were Lsamp and Kif2 show basal expression levels. Beside these described transcription factors yet unknown additional factors may define specific behavioural states more accurate.

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Abstract

The brain depicts a highly plastic and malleable organ, allowing individuals to respond and interact to novel situations and environments. Meaningful experiences will lead to memory acquisition and storage, permitting subjects to reflect upon current situations and allows planning ahead for the future. The auditory cortex represents an excellent model to study plastic changes evoked by memory formation.

It is a crucial question in neuroscience to determine cellular processes involved in a memory task leading to memory formation. Previous studies showed the transcription factor *CREB-regulated transcription coactivator-1* (CRTC1) can act as a sensor for cells, involved in establishing memory. CRTC1 is able to operate as a coincidence detector by sensing increasing intracellular calcium (Ca2+) levels as well as cyclic adenosine monophosphate (cAMP). This induced activity leads to the translocation of CRTC1 from its synapse of a neuron to the nucleus were it binds to *cAMP response element-binding protein* (CREB), achieving the transcription of genes involved in memory formation and a generation of plastic remodeling in the brain. However, most of this information on CRTC1 trafficking was acquired in reduced cell culture systems. Information on the regulation of CRTC1 in the living brain is still scarce. Here, I addressed the question how defined behavioral learning protocols would affect the subcellular translocation of CRTC1.

In my thesis I established an immunohistochemical approach to visualize CRTC1 in sections of the auditory cortex. I showed that CRTC1 expressing cells in the auditory cortex are largely excitatory neurons. I further established a behavioral paradigm to study CRTC1 translocation after neuronal activity. My final results depict a comparison between mice generating an associative memory formation (paired mice) to mice undergoing a non-associative experience (unpaired mice) as well as to a control group represented by naïve mice (home cage mice).

Paired mice and naïve mice showed a significantly higher localization of CRTC1 in the cytoplasm compared to unpaired mice in layer 2/3. Paired mice and naïve mice showed a significant higher localization of CRTC1 in the nucleus compared to unpaired mice in layer 5/6. Nevertheless, substantial variability in CRTC1 location was observed across subjects in the same treatment group. This suggests that behavioral experience may be one, but not the only factor controlling translocation to the nucleus.

In summary, my results provide first evidence that specific behavioral paradigms can induce subcellular translocation of CRTC1 in neurons in vivo. This is interesting in light of previous studies showing that specific experiences can induce distinct global expression patterns in the ACx.

Zusammenfassung

Das Gehirn ist ein äusserst plastisches und modifizierbares Organ. Es erlaubt Individuen mit ihrem Umfeld zu interagieren und ermöglicht ein schnelles Reagieren sowie Adaptieren auf neue Situationen. Subjektiv bedeutungsvolle Erlebnisse werden rasch verarbeitet sowie etabliert was weiters zu einer Festigung im Gedächtnis führt. Individuen sind durch diese Speicherung in der Lage gegenwärtige wie zukünftige Situationen zu überdenken und entstehende Folgen sowie womögliche Gefahren vorher zu sehen. Der auditorische Kortex stellt ein gutes Modell dar um neuronale Aktivitäten sowie die dadurch generierte Gedächtnisformierung, ausgelöst durch externe Reize, experimentell zu untersuchen. Eine wesentliche Frage in der Neurobiologie ist, die zellulären Prozesse zu identifizieren, die einer Gedächtnisformierung zugrunde liegen. Kürzlich erschienenen Studien zeigten die Beteiligung des Transkriptionsfaktors *CREB-regulated transcription coactivator-1* (CRTC1, auch bekannt als TORC1) an der Gedächtnisformierung durch die koinzidente Wahrnehmung zweier wichtiger Signalstoffe: cyclisches Adenosinmonophosphat (cAMP) und Calcium (Ca2+). CRTC1 wandert in Folge durch die intrazelluläre Erhöhung dieser beiden Botenstoffe von einer Synapse in den Zellkern eines Neurons ein und aktiviert dort, über die Bindung an das *cyclic AMP responsible element 1* (CREB1), Gene die bekanntermassen zur Gedächtnisformation beitragen. Ein Grossteil der vorhandenen publizierten Literatur sind Arbeiten welche sich auf Beobachtungen in der Zellkultur stützen. Die in-vivo Situation von CRTC1 hingegen ist relativ unerforscht. Es konnte bis jetzt nur gezeigt werden, dass eine Überexpression im Gyrus dendatus zu einer Gedächtnissteigerung führen kann. Das Ziel meiner Studie ist es daher mittels natürlicher Verhaltensprotokolle, welche eine Gedächtnisformierung induzieren können, die Regulation des subzellulären Transports von CRTC1 im auditorischen Kortex zu untersuchen.

Um CRTC1 in-vivo im auditorischen Kortex nachweisen zu können, habe ich eine immunohistochemische Methode für meine Masterarbeit etabliert. Ich konnte damit nachweisen, dass CRTC1 in Neuronen im auditorischen Kortex vorhanden ist und speziell in exzitatorischen Neuronen eingehende Signale in den Zellkern weiterleitet. Des weiteren analysierte ich im Gewebe des auditorischen Kortex die Lokalisierung von CRTC1 durch den Vergleich dreier Gruppen von Mäusen die unterschiedliche oder kein Gedächtnisformierungsprotokoll durchliefen.

Meine Forschungsergebnisse sind die Folgenden:

Mäuse welche eine Assoziation eines Tones mit einem elektrischen Schock herstellten und dadurch ein Gedächtnis formten, hatten sowie naive Mäuse ein signifikant vermehrtes

Vorhandensein von CRTC1 im Zellkern im auditorischen Kortex Schicht 2/3 verglichen mit Mäusen welche keine Assoziation zwischen Ton und Schock herstellen. Des weiteren hatten Mäuse die eine Assoziation generierten sowie naive Mäuse eine signifikant vermehrtes Vorhandensein von CRTC1 im Zellkern im auditorischen Kortex Schicht 5/6 verglichen mit Mäusen welche keine Gedächtnisformierung herstellen konnten. Trotz dieses signifikanten Trends war eine substantielle Variablilität in der CRTC1 Lokalisation in Präperaten von der gleichen Behandlungsgruppe zu beobachten. Dies deutet darauf hin, dass Verhaltenserfahrungen ein Faktor neben weiteren Faktoren sein kann, die die CRTC1 Lokalisation kontrollieren.

Zusammenfassend lässt sich sagen, dass in dieser Arbeit erstmals Hinweise auf eine subzelluläre Translokation von CRTC1 in Neuronen infolge spezifischer Verhaltensprotokolle gewonnen werden konnten. Die Tatsache, dass bestimmte Transkriptionsfaktoren zufolge bestimmter Verhaltensparadigmen in den Zellkern translozieren ist interessant vor dem Hintergrund, dass diese Paradigmen mit spezifisch globalen Expressionsmustern im auditorischen Kortex korreliert sind.

Curriculum Vitae

Personal Information

Name	Neumayr Christoph
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Education

2013-2014	Master Thesis in Neuroscience Research Institute of Molecular Pathology Group of Dr. Simon Rumpel
2012-2013	Master studies of Neurobiology at the University of Vienna/ Medical University of Vienna
2008 – 2012	Bachelor studies of molecular biology at the University of Vienna Specializations: FISH, light and video microscopy, Microarrays
2002 – 2007	Matriculation at Technologisches Gewerbemuseum in Vienna, Austria

Practical Lab courses, Campus Vienna Biocenter and faculty of chemistry

Date: 2008-2013

Molecular Biology and Genetics I, IIA+B, Microbiology I, II, Cell Biology, Biochemistry
Organic Chemistry Anorganic Chemistry

Lab courses provided the basic skills for molecular work

Internship- Microbiology, University of Natural Resources and Life Science

Supervisor: Sabrina Mayer
Date: August- September 2010

Techniques: Production of solutions
Introduction of HPLC, SDS-PAGE, work with E. coli

Bachelor Thesis, Institute for microbial ecology

Group: Dr. Alexander Loy
Date: Juni 2012

Project Title: *"Identification of sulphate-reducing microorganisms in unknown environmental samples"*
Grade: excellent

Techniques: Microarray
DNA extraction
Bioinformatics analysis

Practical lab course, Center of Brain Research (CBR) – Medical University of Vienna All current departments

Date: March – April 2014

This course provided basic techniques in the field of neuroscience

Techniques:	Cryostat/ Vibratome handling	Brain dissection
	Fluorescence microscope	Patch clamp
	Immunocytochemistry	Receptor binding studies
	MRI	Western blot
	Elispot	

Internship, Institute of Molecular Biotechnology

Group: Dr. Juergen Knoblich

Date: November 2012- February 2013

Project Title: *"Testing of different RBP Jk short hairpin constructs to screen their knockout ability"*

Techniques:
- qPCRs
- Cloning
- PCRs
- Mouse handling
- Cell culture
- Viral injections into mouse embryo brain

Master thesis, Institute of Molecular Pathology

Group:	Dr. Simon Rumpel
Date:	February 2013- September 2014
Project Title:	*"Experience dependent trafficking of the CREB regulated transcription co-activator 1(CRTC1)"*
Techniques:	qPCRs, PCRs Cloning Behavioural experiments Cell culture Histology Stereotaxic injections AAV preparation Confocal microscope

Personal Skills

Language skills:	German – native English – fluent