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Neuronal Activity-Dependent Transcriptional Regulation of the Synaptic Cell Adhesion Molecules *Lrrtm1* and *Lrrtm2*

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Contents

Summary 1

Zusammenfassung 3

Introduction 5

I. Activity-Regulated Gene Expression 5

Neuronal activity, calcium signalling, and gene expression 5

Synaptic activity in learning and memory, and neuronal survival 6

Activity-dependent transcription and human cognition 9

II. Synaptic Cell Adhesion Molecules 11

Functions of synaptic cell adhesion molecules 11

Synaptic cell adhesion molecules and their role in synapse formation, maturation and function 12

Lrrtm proteins in synaptogenesis and mature synapse function 15

Synaptic cell adhesion molecules and neuropsychiatric disorders 17

III. Aim of the Study 19

Material and Methods 20

Cell culture 20

Pharmacological treatments, RNA isolation and quantitative PCR 20

Calcium imaging 21

Immunodetection 22

Luciferase assay 22

AAV vector cloning, virus production and infection 24
Chromatin immunoprecipitation 26
Morphological analyses 27
AMPA receptor trafficking 28
Microelectrode array recordings 30

Results 31

Activity-dependent expression of *Lrrtm1* and *Lrrtm2* 31
Signalling pathways involved in activity-induced expression of *Lrrtm1* and *Lrrtm2* 33
Nuclear calcium signalling regulates the induction of *Lrrtm1* and *Lrrtm2* 36
The role of CBP in *Lrrtm1* and *Lrrtm2* expression 37
Lrrtm1 and *Lrrtm2* expression and CREB 39
The role of c-Jun in *Lrrtm1* and *Lrrtm2* expression 41
Lrrtm1 and *Lrrtm2* expression and MeCP2 43
Influence of *Lrrtm2* knock-down on spine density 46
Influence of *Lrrtm1* and *Lrrtm2* knock-down on network activity 48
Lrrtm1 and *Lrrtm2* and AMPA trafficking 52

Discussion 57

Physiological significance of activity-induced *Lrrtm1* and *Lrrtm2* expression 57
Regulation of genetic modules through the signalling cascade of neuronal activity–nuclear calcium–CREB 60
The role of the co-activator CBP and the modifier MeCP2 in *Lrrtm1* and *Lrrtm2* transcription 61
Experimental considerations 63
Future directions 65

Abbreviations 66

References 69

Summary

The large processing capacity of our brain is the result of properly formed synaptic contacts and their maintenance and plasticity. Synaptic cell adhesion molecules and neuronal activity are critically involved in these processes. While synaptic cell adhesion molecules govern molecular target recognition, structural integrity and plasticity of a synaptic contact, neuronal activity is one major factor underlying dynamic and adaptive responses of neurons and neuronal networks. Neuronal activity and synaptic cell adhesion molecules jointly contribute to the establishment of normal brain function. However, little is known about their interaction and mutual dependence. Here I show that *Lrrtm1* and *Lrrtm2*, two recently identified synaptic cell adhesion molecules, are regulated by neuronal activity in cultured hippocampal neurons of the mouse. I found that their responsiveness to neuronal activity crucially depends on nuclear calcium signalling. In addition *Lrrtm2* is bound and controlled by CREB, an important factor in activity-mediated gene transcription. Using inhibitors of several calcium-dependent pathways, I demonstrate that the expression of *Lrrtm1* and *Lrrtm2* is mediated by calcium/calmodulin-dependent kinases. Further I show that *Lrrtm1* and *Lrrtm2* mRNA levels increase during development, which correlates with the maturation of the neuronal network. I can further show that knock-down of *Lrrtm2* does not influence spine density, contrary to what has been reported in the literature. It does, however, influence neuronal network activity, as I demonstrate in collaboration with H.E. Freitag using microelectrode array recordings. Similar changes appear under *Lrrtm1* knock-down conditions. The network behavior in these cultures reverts to nearly normal by overexpression of Lrrtm1 protein. Similar to the changes observed in *Lrrtm1* and *Lrrtm2* knock-down cultures, overexpression of MeCP2 causes a desynchronization of the neuronal bursting activity. MeCP2 is a transcriptional regulator which is found mutated in Rett syndrome, a rare but severe neurodevelopmental disorder in humans. I could show that the expression of endogenous *Lrrtm2* is deregulated in cultures overexpressing MeCP2. This suggests that the network changes observed in MeCP2 overexpressing cultures are caused by deregulation of *Lrrtm2* by MeCP2. However, overexpression of Lrrtm2 protein failed to rescue the MeCP2-phenotype. A further aim of my studies was to analyze the function of the activity-responsiveness of

Lrrtm1 and *Lrrtm2*. Using different methods I attempted to visualize AMPA receptor trafficking to the neuronal surface and the impact of *Lrrtm* knock-down thereon. However, the applied methods were too insensitive to detect changes in synaptic AMPA receptor surface expression.

Together, these findings connect *Lrrtm1* and *Lrrtm2*, respectively, two members of the group of synaptic cell adhesion molecules, to synaptic activity, nuclear calcium signalling and CBP/CREB, all of which are important mediators of sustained adaptive changes in the central nervous system. The findings also give the impetus to further explore the role of the *Lrrtm1* and *Lrrtm2* activity-responsiveness in neurons *in vitro* and *in vivo*.

Zusammenfassung

Die Fähigkeit unseres Gehirns, Signale aufzunehmen und zu verarbeiten basiert auf der korrekten Bildung synaptischer Kontakte sowie deren Aufrechterhaltung und Plastizität. An diesen Prozessen maßgeblich beteiligt sind synaptische Zelladhäsionsmoleküle und neuronale Aktivität. Während synaptische Zelladhäsionsmoleküle die Erkennung, Struktur und Plastizität synaptischer Kontakte steuern, ist die neuronale Aktivität eine der Hauptkomponenten dynamischer und adaptiver Prozesse in Neuronen und neuronalen Netzwerken. Beide tragen gemeinsam dazu bei, eine einwandfreie Hirnfunktion sicherzustellen. Es ist jedoch wenig darüber bekannt, wie sie sich gegenseitig beeinflussen und miteinander interagieren. In diesem Projekt zeige ich, dass die vor kurzem entdeckten synaptischen Adhäsionsmoleküle *Lrrtm1* und *Lrrtm2* durch neuronale Aktivität reguliert werden. Diese aktivitätsabhängige Steuerung wird von im Zellkern lokalisierten Calciumsignalen vermittelt. *Lrrtm2* wird zusätzlich durch CREB, einen wichtigen Faktor der aktivitätsabhängigen Gentranskription, gebunden und kontrolliert. Durch die Inhibition verschiedener Calcium-vermittelter Signalwege demonstriere ich, dass die Expression von *Lrrtm1* und *Lrrtm2* von Calcium-/Calmodulin-abhängigen Kinasen gesteuert wird. Weiter kann ich zeigen, dass die mRNA Level von *Lrrtm1* und *Lrrtm2* während der Entwicklung ansteigen und damit in Korrelation mit der Ausbildung des neuronalen Netzwerkes stehen. Ferner konnte ich zeigen, dass der Knock-down von *Lrrtm2*, anders als in der Literatur beschrieben, keinen Einfluss auf die Dichte dendritischer Spines hat. Er beeinflusst jedoch die anhand von Mikroelektroden-Arrays gemessene neuronale Netzwerkaktivität, was ich in Kollaboration mit H.E. Freitag nachgewiesen habe. Ähnliche Veränderungen kann man bei einem *Lrrtm1* Knock-down beobachten. In diesen Kulturen normalisiert sich die Netzwerkaktiviät nahezu komplett, wenn Lrrtm1 Protein überexprimiert wird. Zu den Knock-downs von *Lrrtm1* bzw. *Lrrtm2* vergleichbare Veränderungen finden sich auch, wenn MeCP2 überexprimiert wird; es verursacht eine Desynchronisation der Netzwerkaktivität. MeCP2 ist ein Transkriptionsregulator, welcher beim Rett Syndrom mutiert ist, einer seltenen aber schweren neurologischen Entwicklungsstörung des Menschen. Ich konnte außerdem zeigen, dass die Expression von endogenem *Lrrtm2* durch Überexpression von MeCP2 erniedrigt wird. Dies legt nahe, dass die

Netzwerkveränderungen, die unter MeCP2 Überexpression zu beobachten sind, durch die Abnahme der endogenen *Lrrtm2* Level hervorgerufen werden. Die Überexpression von Lrrtm2 Protein ist jedoch nicht ausreichend, um den ursprünglichen Phänotyp wiederherzustellen. Ein weiteres Ziel meiner Doktorarbeit war es, die physiologische Funktion der aktivitätsabhängigen *Lrrtm1* und *Lrrtm2* Induktion zu erforschen. Mit Hilfe verschiedener Methoden habe ich versucht, den AMPA-Rezeptortransport zur Synapsenoberfläche und die Wirkung eines *Lrrtm* Knock-downs hierauf zu detektieren.

Zusammengenommen zeigen die Ergebnisse dieses Projektes eine Verbindung zwischen den Genen der synaptischen Adhäsionsmoleküle *Lrrtm1* und *Lrrtm2* und neuronaler Aktivität, nukleären Calciumsignalen sowie CBP bzw. CREB. All diese Faktoren gelten als wichtige Mediatoren von langfristigen adaptiven Veränderungen im zentralen Nervensystem. Die Ergebnisse legen außerdem den Grundstein zur weiteren Erforschung der Bedeutung der aktivitätsabhängigen Regulation von *Lrrtm1* und *Lrrtm2* in Neuronen *in vitro* und *in vivo*.

Introduction

I. Activity-Regulated Gene Expression

Neuronal activity plays a crucial role in initiating a multitude of events that, in their entirety, lead to normal brain function. In order for a neuron to exert its proper role in this process, it is required to dynamically respond to external stimuli. One of the essential parts of this adaptive responsiveness is gene expression regulated by neuronal activity.

Neuronal activity, calcium signalling, and gene expression

The basic function of a synapse is to connect neurons and propagate electric signals – this is achieved by presynaptic neurotransmitter release and subsequent receptor activation and influx of ions into the postsynaptic neuron, leading to depolarisation of the neuronal membrane (Bishop and McLeod, 1954; Curtis and Eccles, 1958; Burton, 1966). In parallel to this global function, a number of processes are initiated that cause internal changes in the postsynaptic neuron. These include regulation of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors (Malinow and Malenka, 2002), control of actin cytoskeleton dynamics (Fischer et al., 1998; Matus, 2000), initiation of local protein synthesis (Steward and Levy, 1982; Steward and Schuman, 2001) and activation of gene transcription (Greenberg et al., 1985; Greenberg et al., 1986). The latter, gene transcription due to synaptic activity, critically depends on the mechanism of calcium-mediated signalling.

Neuronal sources of calcium are manifold; it can enter the cytosol from the outside of the cell through voltage-dependent calcium channels, NMDA (*N*-methyl-D-aspartate) receptors, and AMPA receptors, and from intracellular release stores such as IP3- and ryanodine receptor-dependent calcium stores (Bito, 1998). The most studied source of cytosolic calcium in the context of gene transcription are NMDA receptors (Cole et al., 1989; Szekely et al., 1990; Bading et al., 1995; Xia et al., 1996). These receptors are cation

channels gated by glutamate, one of the main excitatory neurotransmitters in the central nervous system, and are activated in a magnesium/voltage-dependent manner (Nowak et al., 1984). The channels are permeable to sodium, which contributes to postsynaptic depolarization, and calcium, which generates intracellular calcium transients (MacDermott et al., 1986). The heterogeneity of calcium signalling-dependent effects is accomplished by the creation of calcium micro-domains (Hardingham et al., 2001a, b) and by the activation of various calcium-binding proteins (Kasai, 1993). These proteins have different calcium binding capacities and vary in their cellular localization, suggesting that each calcium binding protein possesses specific functions (Kasai, 1993; Bito, 1998). One prominent calcium-binding protein is calmodulin: it is abundant in neurons, both in the cytoplasm and the nucleus and upon binding of calcium it activates a large variety of calcium/calmodulin (CaM)-dependent effector molecules (Klee, 1991; Means et al., 1991; Bito, 1998). Calcium transients, especially those propagating to the nucleus (nuclear calcium signalling (Hardingham et al., 1997; Hardingham et al., 2001b)), and CaM trigger a range of intracellular signalling pathways that ultimately lead to the activation of gene transcription (Bito, 1998; Greer and Greenberg, 2008). They include the Ras/MAPK/ERK pathway, CaM-dependent kinase signalling (e.g. CaMKII and CaMKIV), calcineurin and p38 MAP kinases (Westphal et al., 1998; Kasahara et al., 1999; Dolmetsch et al., 2001; Wheeler et al., 2008).

All these pathways result in the activation of transcription factors and transcription-modulating proteins, and thus, in altered gene expression. The nature of the modified genetic programme defines the functional outcome of neuronal activity and leads to long-term adaptive changes like memory formation and cell survival.

Synaptic activity in learning and memory, and neuronal survival

Learning and memory require neuronal activity, and the long-term storage of information depends on activity-mediated gene transcription. In a similar way, neuronal activity renders neurons more resistant to toxic conditions by activating distinct genes. Thus, memory formation and neuronal survival find their common denominator in synaptic activity, and activity-mediated gene transcription.

LTP (long-term potentiation) is a neuronal mechanism thought to be a prerequisite of learning and memory (Teyler and Discenna, 1984). It describes the increase in synaptic strength as a result of a specific electric stimulus (Bliss and Lomo, 1973). The late phase of LTP requires gene transcription (Nguyen et al., 1994) and one of the pathways that has been shown to be involved in conveying the signal from the synapse to the nucleus in LTP and memory consolidation is the MAPK pathway. It has been demonstrated that theta burst stimulation induces the production of cAMP, which activates MAPK and leads to the release of BDNF. BDNF in turn activates the TrkB receptor on the cell surface, which leads to the translocation of MAPK to the nucleus, where it gains access to transcription factors (Patterson et al., 2001; Kelly et al., 2003).

A prominent example of a transcription factor shown to be involved in LTP and memory formation is CREB (cAMP/calcium response element-binding protein) (Bourtchuladze et al., 1994; Yin et al., 1994; Ahn et al., 1999; Barth et al., 2000; Josselyn et al., 2001; Josselyn et al., 2004; Pham et al., 2004). CREB phosphorylation and the initiation of CRE (cAMP/calcium response element)-controlled gene expression are triggered during training in hippocampus-dependent tasks (Impey et al., 1998; Taubenfeld et al., 1999). Additionally, mutant mice expressing a dominant negative form of CREB, KCREB, demonstrate deficiencies in different forms of LTP and learning (Pittenger et al., 2002; Barco et al., 2006). In addition to CREB, other transcription factors have been found implicated in transcription-dependent LTP and learning and memory, for example, serum response factor, c-fos, and NFκB (Tischmeyer and Grimm, 1999; Albensi and Mattson, 2000; Ramanan et al., 2005).

A large number of genes shows altered expression in the brain after learning (Cavallaro et al., 2001; Luo et al., 2001; Cavallaro et al., 2002; Leil et al., 2003) pointing to the possibility that global chromatin alteration is involved in memory formation. This includes epigenetic modifications of DNA and DNA-organising proteins, like DNA methylation and histone acetylation, respectively (Levenson and Sweatt, 2005). Acetylation of histone H3, for example, is increased by long-term memory formation (Levenson et al., 2004). In addition, mice expressing an inducible dominant-negative form of CBP (CREB-binding protein), a transcriptional co-activator and histone acetyltransferase (Kalkhoven, 2004), show impaired learning in the spatial water maze task and novel object recognition

(Korzus et al., 2004). Exposure of these CBP-deficient animals to a histone deacetylase inhibitor restores normal long-term memory formation (Alarcon et al., 2004; Korzus et al., 2004). Similarly, *DNA methyltransferase 3A* and *3B* gene expression is upregulated in the hippocampus of rats following contextual fear conditioning, and inhibition of the methyltransferase abolishes long-term memory formation (Miller and Sweatt, 2007).

In addition to being the trigger for memory formation and learning, neuronal activity supports the initiation of a pro-survival gene expression programme. Synaptically evoked bursts of action potentials lead to an influx of calcium through NMDA receptors. These calcium transients trigger two signalling pathways, both resulting in the phosphorylation of the transcription factor CREB: the MAPK pathway and the CaM kinase pathway (Bading and Greenberg, 1991; Chawla et al., 1998). While MAPK is activated in the vicinity of the NMDA receptor by cytosolic calcium and is responsible for a prolongation of CREB phosphorylation beyond synaptic activity (Hardingham et al., 2001a; Impey and Goodman, 2001; Wu et al., 2001), the CaM kinase pathway requires the calcium signal to propagate to the nucleus, resulting in phosphorylation and activation of CREB and its transcriptional co-activator CBP within seconds of calcium influx (Chrivia et al., 1993; Hardingham and Bading, 2003). This nuclear calcium signal turned out to be the key mediator of CREB-induced neuroprotective gene transcription (Lee et al., 2005; Papadia et al., 2005; Zhang et al., 2009; Zhang et al., 2011; Tan et al., 2012). Nine genes were identified as strong promoters of neuronal survival; they include *Atf3*, *Btg2*, *GADD45β*, *GADD45γ*, *Inhibin β-A*, *Ifi202b*, *Npas4*, *Nr4a1*, and *Serpinb2* (Zhang et al., 2009; Zhang et al., 2011; Tan et al., 2012). Several of the genes provide neuroprotection by rendering mitochondria more resistant to cellular stress (*Inhibin β-A*, *Ifi202b, Npas4*, *Nr4a1*), while others confer neuroprotection by regulating a subset of genes through yet unknown means (Zhang et al., 2009; Zhang et al., 2011). The activity-induced transcriptional repressor Atf3, for example, controls a genetic module that protects against ischemic brain damage (Zhang et al., 2011).

Hence, starting from a single stimulus –synaptic activity– our brain cells exploit their versatile options by subdividing this stimulus into several pathways and finally the expression of distinct genes.

Activity-dependent transcription and human cognition

Neurodevelopmental disorders in humans are characterised by symptom-onset in early postnatal life. In this period, activity-dependent gene transcription is highly induced, which raises the possibility that defects in activity-dependent gene expression play a role in the aetiology of human cognitive disorders (Greer and Greenberg, 2008). This is supported by the finding that mutations in the components that convey the activity-induced signal from the synapse to the nucleus cause cognitive disorders (Greer and Greenberg, 2008). These disorders comprise Timothy syndrome, Coffin-Lowry syndrome, Rubinstein-Taybi syndrome, Rett syndrome and autism (Petrij et al., 1995; Amir et al., 1999; Hanauer and Young, 2002; Splawski et al., 2004; Greer and Greenberg, 2008; Morrow et al., 2008).

Two of these syndromes are of particular interest in the context of this project: Rubinstein-Taybi syndrome and Rett syndrome.

Rubinstein-Taybi syndrome is a condition characterized by moderate to severe learning deficits and mental disability, as well as typical physical features including facial abnormalities, broad thumbs, big toes and a short statue (Rubinstein and Taybi, 1963; Petrij et al., 1995). Many patients with Rubinstein-Taybi syndrome have breakpoints and microdeletions in the region of chromosome 16pl3.3 (Imaizumi and Kuroki, 1991; Breuning et al., 1993; Masuno et al., 1994); in 1995, Petrij *et al*. identified in this region the *CBP* gene as the critical factor causing the syndrome (Petrij et al., 1995). CBP is a co-activator of many transcription factors (Shiama, 1997), including CREB, which is an important regulatory element for activity-dependent adaptive changes in neurons (Impey et al., 1998; Barth et al., 2000; Zhang et al., 2011). In addition, CBP has histone acetyltransferase activity (Kalkhoven, 2004) and is implicated in posttranslational modification of histones during learning (Korzus et al., 2004). Therefore it is comprehensible that patients lacking functional CBP suffer from cognitive disabilities.

Patients with Rett syndrome present a different spectrum of symptoms: children develop normally until the age of one to two years, after which their development halts and eventually regresses, with the evolvement of repetitive hand movements, gait problems, autistic features, and severe mental disabilities (Rett, 1966; Samaco and Neul, 2011). A gene found to be mutated in individuals with Rett syndrome is *MECP2* (Amir et al., 1999).

It encodes a protein that is abundantly expressed in the nucleus of neurons and is implicated in methylation-dependent transcriptional regulation, although its exact function remains elusive (Chahrour et al., 2008; Skene et al., 2010; Mellen et al., 2012). It has been shown, however, that MeCP2 is phosphorylated upon neuronal activity, suggesting that it exerts its function, at least partly, in an activity-dependent manner (Zhou et al., 2006; Cohen et al., 2011).

The above described mechanisms underlying memory formation and neuronal survival are only two examples of activity-induced and transcription-dependent adaptive changes in neurons. In order to fully understand the role of activity-regulated gene expression under physiological and pathophysiological conditions it is necessary to identify the genes that change their expression upon synaptic activity. In a second, much more laborious step, the function of these genes has to be studied. In my PhD project, I analysed the activity-dependent transcription of two genes, *Lrrtm1* and *Lrrtm2*, thereby identifying them as part of the activity-regulated genetic programme in neurons. The second step however, elucidating their function, remains to be uncovered. Yet, since Lrrtm1 and Lrrtm2 belong to the family of synaptic cell adhesion molecules, knowing the functions of these molecules might help to understand those of *Lrrtm1* and *Lrrtm2*.

II. Synaptic Cell Adhesion Molecules

Brain function arises from the appropriate connectivity of neurons, with the synaptic contact as the essential part of this connectivity. Synapses are asymmetric neuronal connections with distinct molecules found on the pre- and postsynaptic side, separated by the synaptic cleft. The formation of a functional synaptic contact requires the precise alignment of the presynaptic neurotransmitter release machinery and the postsynaptic receptor apparatus. This initial formation of a synaptic junction is followed by maturation and plasticity of the established synapse. In all of these processes one class of proteins plays a major role: synaptic cell adhesion molecules.

Functions of synaptic cell adhesion molecules

Synaptic cell adhesion molecules are transmembrane proteins anchored in the synaptic membrane of neurons. They engage in hetero- or homophilic interactions across the synaptic cleft and organize synaptic components on both sides of the membrane. Based on their nature, four functions can be established for these proteins (Yamagata et al., 2003).
The first is target recognition: after axons have been guided to their target region, they need to choose the correct binding partner. The recognition of the correct partner in the enormous network of neuronal processes, and the precise location of the connection on a certain part of the dendrite, can be achieved by specific protein-protein interaction of pre- and postsynaptic adhesion molecules (Dalva et al., 2000; Yamagata and Sanes, 2008; Pecho-Vrieseling et al., 2009).
Following the initial contact of two neurons, a stable synaptic junction is formed; this is the second and presumably most apparent function of synaptic cell adhesion molecules. They maintain the integrity of the synaptic contact and guarantee its stability by attaching the pre- and postsynaptic membranes (Gray and Whittaker, 1962; Yamagata et al., 2003).
As a third function, synaptic cell adhesion molecules influence synapse maturation and differentiation. The adhesion proteins allow a bidirectional signalling between neurons that form a synapse. Expression of presynaptic proteins induces postsynaptic differentiation in

contacting neurons and vice versa, leading to the recruitment of the appropriate signalling machinery on either side (Scheiffele et al., 2000; Graf et al., 2004). In addition, the nature of the adhesion molecule dictates the type of synapse formed: e.g. distinct forms of adhesion molecules specifically bind to partners exclusively found at inhibitory or excitatory synapses (Graf et al., 2004; Chih et al., 2006).

Finally, synaptic cell adhesion molecules are able to affect synaptic function. Synaptic plasticity, i.e. the dynamic modulation of the strength of the synaptic signal, is thought to be the cellular correlate of learning and memory. One form of synaptic plasticity is long-term potentiation (LTP), where a certain stimulus leads to a sustained increase in synaptic strength (Bliss and Lomo, 1973). This plasticity was found to be influenced by synaptic cell adhesion molecules (Luthl et al., 1994; Muller et al., 1996; Yamagata et al., 1999; Bozdagi et al., 2000). Thus, in addition to their contribution to synapse function by mere provision of scaffolding structures for synaptic vesicles and corresponding neurotransmitter receptors, synaptic cell adhesion molecules also directly influence physiological processes at the synapse.

Synaptic cell adhesion molecules and their role in synapse formation, maturation and function

The great diversity of synaptic cell adhesion molecules includes integrins, cadherins, neuroligins, neurexins, nectin-like synaptic cell adhesion molecules (SynCAMs), synaptic adhesion-like molecules (SALMs), netrin-G-ligands (NGLs), leucine-rich repeat transmembrane proteins (Lrrtms), ephrin receptors (Eph) and Sidekicks (Tallafuss et al., 2010). Up to now, no single pair of synaptic cell adhesion molecules was found to be sufficient to organize all aspects of synapse formation and function, indicating that synaptic cell adhesion molecules most likely have overlapping functions and co-operate at synapses (Dalva et al., 2007).

The prototypic synaptic cell adhesion complex and classic example of bidirectional synaptic signalling is the interaction between presynaptic neurexins and postsynaptic neuroligins (Ushkaryov et al., 1992; Ichtchenko et al., 1995; Ichtchenko et al., 1996; Nguyen and Sudhof, 1997; Song et al., 1999). In rodents, four neuroligins exist

(neuroligin1-4) (Ichtchenko et al., 1995; Ichtchenko et al., 1996; Hoon et al., 2011); they all terminate in a PDZ domain binding site and can bind to the post-synaptic density protein 95 (PSD95) (Irie et al., 1997). Following recruitment of PSD95, functional AMPA receptors accumulate by trapping membrane-diffusible AMPARs, presumably through interaction of the neuroligin-PSD95 complex with the protein stargazin (Bats et al., 2007; Heine et al., 2008; Mondin et al., 2011). Whether this recruitment of AMPA receptors requires neuronal activity is not entirely resolved (Nam and Chen, 2005; Chubykin et al., 2007; Heine et al., 2008). NMDA receptors are also recruited to clusters of neuroligins at the postsynaptic site, most likely through extracellular interactions (Graf et al., 2004; Chih et al., 2005; Budreck et al., 2013). The synaptogenic capacity of neuroligins was first discovered in a co-culture assay, where expression of neuroligin in non-neuronal cells induced presynaptic differentiation in contacting neurons (Scheiffele et al., 2000). This accumulation of presynaptic components is mediated by the interaction of neuroligin with neurexins (Scheiffele et al., 2000; Dean et al., 2003), whereas binding of the two partners occurs in an iso- and spliceform specific manner and defines the nature of the synapse: while neuroligin1 primarily binds to neurexin1β and localizes to excitatory synapses, neuroligin2 interacts with neurexin1α and is found at inhibitory synapses (Ichtchenko et al., 1995; Ichtchenko et al., 1996; Chih et al., 2005; Chih et al., 2006; Chubykin et al., 2007). On the presynaptic side, neurexins recruit synaptic vesicles via CASK (calmodulin-dependent serine protein kinase) and MINT (Munc 18 interacting protein) (Hata et al., 1996; Butz et al., 1998; Biederer and Sudhof, 2000) and couple calcium channels to synaptic vesicle exocytosis (Missler et al., 2003). Taken together, the neuroligin-neurexin interaction accounts for important parts of pre- and postsynaptic differentiation; nevertheless, many more functions can be attributed to synaptic cell adhesion molecules in synaptogenesis and synapse function.

Another important bidirectional signalling complex at the synapse is the contact between the receptor tyrosine kinase EphB and its ligand, ephrin-B. Primarily known as axon guidance molecules (Huot, 2004), EphB receptors and their ligands, ephrin-Bs, have in the past decade received much attention for their role in synaptogenesis and mature synapse function (Torres et al., 1998; Dalva et al., 2007; Sloniowski and Ethell, 2012). It has been shown that clustering and activation of EphB at the postsynaptic side with a soluble ephrin-

B-Fc fusion protein induces the formation of dendritic spines, recruitment of NMDA and AMPA receptors, and triggers differentiation of presynaptic terminals (Dalva et al., 2000; Henkemeyer et al., 2003; Kayser et al., 2006; Dalva et al., 2007). Upon activation by ephrin-B, EphBs are able to induce downstream signalling pathways through the GEFs (guanine nucleotide exchange factors) Tiam1 and kalirin, resulting in spine formation and morphogenesis (Penzes et al., 2003; Tolias et al., 2007) as well as the transition of dendritic filopodia to stable spine structures via kalirin–PAK (p21 activated kinase) signalling (Kayser et al., 2008; Tallafuss et al., 2010).
Most intriguing, however, are the findings about the role of EphBs and ephrin-B in mature synapse function and in synaptic plasticity. LTP at hippocampal mossy fibre-CA3 synapses was shown to implicate transsynaptic signalling between postsynaptic EphBs and presynaptic ephrinBs (Contractor et al., 2002; Armstrong et al., 2006). Three components of this interaction were essential for the induction of LTP; on the postsynaptic side, disruption of the interaction between EphB and PDZ binding domains inhibited LTP (Contractor et al., 2002). On the presynaptic side, abrogation of ephrin-B intracellular signalling by replacing its cytoplasmic terminal with a nonfunctional domain also resulted in reduced LTP (Armstrong et al., 2006). Finally, blocking extracellular EphB–ephrin-B interaction by application of a soluble ephrin-B-Fc fusion protein decreased LTP as well (Contractor et al., 2002).

Many other synaptic cell adhesion molecules have been found implicated in synaptic plasticity by striking and very different mechanisms (Finne et al., 1983; Becker et al., 1996). However, a detailed description would be beyond the scope of this introduction.

In summary, there are several lines of evidence suggesting that synaptic cell adhesion molecules influence and regulate synaptic plasticity and thus influence brain function. The existing multitude of adhesion proteins and mechanisms fuels the notion that many functions of these molecules are still to be uncovered. This notion is underlined by the recent discovery of a new member of the synaptic cell adhesion protein family, Lrrtms (leucine-rich repeat transmembrane proteins).

Lrrtm proteins in synaptogenesis and mature synapse function

In 2009, Linhoff *et al.* identified a new group of synaptogenic proteins, the Lrrtm protein family. The family consists of four members (Lrrtm1-4), all of which are able to induce presynaptic differentiation by trans-cellular signalling in co-culture assays with non-neuronal cells (Linhoff et al., 2009). Lrrtm1 and Lrrtm2 demonstrate the most potent synaptogenic activity (Linhoff et al., 2009). Localized at the postsynaptic side, they induce presynaptic clustering of synapsin and VGLUT1 (vesicular glutamate transporter 1); accordingly, they are found at excitatory synapses (de Wit et al., 2009; Ko et al., 2009; Linhoff et al., 2009). Further, they were found to bind the presynaptic proteins neurexin1α and β (de Wit et al., 2009; Ko et al., 2009). Lrrtm1 and Lrrtm2 specifically bind α and β neurexins lacking an insert at splice site 4 (Ko et al., 2009; Siddiqui et al., 2010).

In vitro experiments in cultured rat hippocampal neurons demonstrated that overexpression of full-length Lrrtm2 increases synapse density (de Wit et al., 2009; Ko et al., 2009). The synaptogenic activity is abrogated by removal of the extracellular domain, indicating that binding to neurexin is required for synapse formation (de Wit et al., 2009; Linhoff et al., 2009). In contrast, RNA interference experiments leading to knock-down of *Lrrtm2* did not decrease synapse numbers (Ko et al., 2011). A decrease in synapse density was only observed in concurrent knock-downs of *Lrrtm1, Lrrtm2,* and *neuroligin3* in neurons cultured from *neuroligin1* knock-out mice; consequently, all four proteins need to be downregulated in order to see an effect on synapse numbers (Ko et al., 2011). Intriguingly, this decrease in synapse density required synaptic activity: chronic treatment with a combination of neurotransmitter receptor inhibitors (APV, NBQX, LY341495) blocked the synapse loss in *Lrrtm1*/*Lrrtm2*/*neuroligin3* triple knock-down neurons cultured from *neuroligin1* knock-out mice (Ko et al., 2011). Detailed analysis revealed that the activity-dependent synapse loss depends on active AMPA receptors, calcium influx, and CaM-dependent kinase activity (Ko et al., 2011). These results are accordable with a model where synapses are continuously eliminated and reformed in an activity-dependent manner, with this 'proof-reading' mechanism requiring neuroligins and Lrrtms (Ko et al., 2011). Additionally, these findings indicate a high degree of redundancy between the Lrrtms and the neuroligins concerning their role in synapse formation *in vitro* (Ko et al.,

2011). However, this redundancy was not observed in regard to electrophysiological properties. *In vivo* experiments in mouse hippocampal slice cultures showed a decrease in AMPA receptor EPSC (evoked excitatory postsynaptic currents) at P14-18 for *Lrrtm1/Lrrtm2* double knock-down beginning at P0 (Soler-Llavina et al., 2011). This effect was rescued by overexpression of Lrrtm2 (Soler-Llavina et al., 2011). Knock-down of *Lrrtm1, Lrrtm2*, and *neuroligin3* in *neuroligin1* knock-out mice further reduced AMPA receptor EPSCs; in addition, NMDA receptor EPSCs were decreased under these conditions (Soler-Llavina et al., 2011). However, this role of Lrrtms in synaptic function seems to be restricted to synaptogenesis as these findings are only apparent during the first two postnatal weeks: introduction of the *Lrrtm1*/*Lrrtm2* double knock-down at later stages (P21) has no effect on the NMDAR/AMPAR ratio (Soler-Llavina et al., 2011).

Morphological analysis of brains from *Lrrtm1* knock-out mice revealed a reduction in hippocampus volume, an increase in hippocampal spine length and in the mean inter-vesicular distance in both the stratum radiatum and the stratum oriens (Takashima et al., 2011). Similarly, Linhoff *et al*. described a selective increase in the size of VGLUT1 puncta in the same regions, stratum radiatum and stratum oriens, determined by immunofluorescence analysis (Linhoff et al., 2009). To date, there is no description of a *Lrrtm2* knock-out mouse. However, Soler-Llavina *et al*. performed RNAi-mediated knock-down of *Lrrtm1*/*Lrrtm2* and *neuroligin3* in hippocampal slice preparations from *neuroligin1* knock-out mice starting at P0, and reported no significant changes in spine density (Soler-Llavina et al., 2011); this also contrasts the results obtained from cultured neurons, where the knock-down leads to synapse loss (Ko et al., 2011; Soler-Llavina et al., 2011).

Together, these findings underline the complex role of Lrrtms and synaptic cell adhesion molecules in synaptogenesis and mature synapse function; as a consequence, many aspects have to be reconciled by taking a broader point of view in order to obtain a coherent and logic picture of synaptic connectivity.

Synaptic cell adhesion molecules and neuropsychiatric disorders

Research in the last decade has made major contributions to our understanding of synaptic cell adhesion molecules and their roles in synaptogenesis and mature synapse function, a small fraction of which is described above. Extrapolation of these findings to neurological and neuropsychiatric disorders and vice versa will help us to understand disease mechanisms and specific functions of molecules. Several synaptic cell adhesion molecules are implicated in neurological and neuropsychiatric disorders, including, but not limited to, autism (cadherin, neurexin, neuroligin, SynCAM), schizophrenia (cadherin, Lrrtm, neurexin), intellectual disability (cadherin, Lrrtm), and anxiety (EphB) (Sudhof, 2008; Siddiqui and Craig, 2011; Redies et al., 2012; Reichelt et al., 2012; Sheffler-Collins and Dalva, 2012).

Neurexins were among the first synaptic cell adhesion molecules found to be implicated in neurodevelopmental disorders (Feng et al., 2006; Szatmari et al., 2007; Kim et al., 2008). Several different neurexin1 mutations have been identified in patients with autism, a disorder characterized by impaired social interaction and communication, and by restricted and repetitive behaviour (American Psychiatric Association, 2000). These mutations were all heterozygous and included point mutations, translocations, and large-scale deletions in the *neurexin1* gene (Feng et al., 2006; Szatmari et al., 2007; Kim et al., 2008; Marshall et al., 2008; Yan et al., 2008; Zahir et al., 2008). The majority of these mutations affected *neurexin1α*, a gene that shares the chromosomal location with *neurexin1β*, but is controlled by a different promoter. Etherton *et al.* estimated that 0.5% of all autism spectrum disorder cases harbour *neurexin1α* gene deletions (Etherton et al., 2009).

Mutations affecting neurexins have also been found in patients suffering from schizophrenia. Schizophrenia is a chronic illness characterized by perturbations in cognition, affect and behaviour, with delusions and auditory hallucinations (American Psychiatric Association, 2000). The *neurexin* mutations found in schizophrenia patients were mainly deletions and affected the promoter and initial exons of the *neurexin1α* gene (Kirov et al., 2008; Vrijenhoek et al., 2008; Walsh et al., 2008; Need et al., 2009). Approximately 0.16% of all schizophrenia cases have *neurexin1α* deletions (Kirov et al.,

2009), while the frequency of these deletions in the normal control population ranges between 0.019% and 0.02% (Kirov et al., 2009; Ching et al., 2010).

Since neurexin1 was identified as the presynaptic binding partner of Lrrtms, disorders with mutation in neurexin1 might also arise from their failed interaction, resulting in an impairment of transsynaptic Lrrtm function, and vice versa.

Due to their comparatively recent discovery, knowledge about the role of Lrrtms in human cognitive disorders is limited. There are, however, several interesting case reports and mouse model studies concerning Lrrtm1, Lrrtm2 and Lrrtm3. For *LRRTM1,* a three-marker SNP (single nucleotide polymorphism) haplotype upstream of the gene was found to be associated with schizophrenia when inherited paternally (Francks et al., 2007; Ludwig et al., 2009). In addition, data was provided suggesting that *LRRTM1* might make a general contribution to handedness (Francks et al., 2007; Ludwig et al., 2009). Further, in a case report of a 9-year-old girl with a *de novo* interstitial deletion on chromosome 2, *LRRTM1* was one of the deleted genes (Rocca et al., 2012). The girl presented a mild intellectual disability with difficulties of sentence organization, understanding, and ability in reading and writing (Rocca et al., 2012). Mice that lack *Lrrtm1* perform differently in behavioral tasks compared to wild-type mice: they avoid approaching to large inanimate objects, have social discrimination deficits and present difficulties in spatial memory (Takashima et al., 2011). Voikar *et al*. described another *Lrrtm1* knock-out mouse with a phenotype of avoiding small enclosures, which was proposed as a mouse model for claustrophobia (Voikar et al., 2013).

In a case report, a 7-year-old boy with a microdeletion on chromosome 5 was described with intellectual disability, developmental delay and mild dysmorphic features (Kleffmann et al., 2012). The mutation was a *de novo* heterozygous microdeletion in 5q31.2 and affected nine genes, one of which was *LRRTM2* (Kleffmann et al., 2012). Similar microdeletions have been described in seven other patients; all displayed facial anomalies and developmental delay, and some individuals were affected by speech delay, short stature and muscular hypotonia (Mosca et al., 2007; Rosenfeld et al., 2011; Shimojima et al., 2011; Kleffmann et al., 2012).

Finally, genetic variants of *LRRTM3* were found to be associated with a risk for late-onset Alzheimer's disease: five single-nucleotide polymorphisms in the promoter region and in intron 2 were found to correlate with the disease (Reitz et al., 2012).

III. Aim of the Study

Both activity-regulated gene expression and synaptic cell adhesion molecules are areas of intense research that made major contributions to our understanding of how the brain works. However, despite the in-depth knowledge in these fields, little is known about their interaction and reciprocal influence. While it is obvious that synapses –and thus, synaptic cell adhesion molecules– are required to induce activity-dependent gene expression, it is less apparent how this regulated gene expression influences the synapse and its components. Therefore I want to study one possible mechanism of how activity-dependent gene expression might contribute to synapse function: by regulating the genes encoding synaptic cell adhesion molecules. Lrrtm1 and Lrrtm2 belong to this group of molecules and are important proteins for the integrity of basal synaptic transmission in early postnatal life. I want to find out whether and how neuronal activity regulates their corresponding genes, *Lrrtm1* and *Lrrtm2*, and explore the function of their activity-responsiveness. Thereby I want to contribute to both the understanding of the function of activity-regulated gene expression and of synaptic cell adhesion molecules.

Material and Methods

Cell culture

Hippocampal neurons from new-born C57Black mice were plated on poly-D-lysin/laminin (PDL/LA; Sigma)-coated culture dishes (diameter 35mm) at a density of 0.39 hippocampi per 1ml Neurobasal media (NBA; Invitrogen) containing 1% rat serum and B27 (Invitrogen). For inhibition of glial cell growth cytosine-1-β-D-arabinofuranose [2.7µM] (Sigma) was added to the culture medium at day *in vitro* 3. At day *in vitro* 8 medium was changed to Transfection medium (TM) containing Salt Glucose Glycine solution (SGG; [140.1mM NaCl, 5.3mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$, 10mM HEPES, 1mM glycine, 30mM glucose, and 0.5mM sodium pyruvate]) (Bading et al., 1993) supplemented with Minimum Essential Medium (MEM, with Earle's salt, without L-glutamine) (Invitrogen), Insulin-transferrin-sodium selenite media supplement [6.3µg/ml-5.7µg/ml-7.5µg/ml] (Sigma), and Penicillin/Streptomycin solution [1:200] (Sigma) unless otherwise stated. Following the medium change on day *in vitro* 8, half of the medium was changed every second day to provide a continuous supply of growth and trophic factors.

Pharmacological treatments, RNA isolation and quantitative PCR

Pharmacological treatments were done after a culturing period of 10 to 12 days *in vitro* during which hippocampal neurons express functional glutamate receptors (NMDA/AMPA/kainate) and develop a rich network of synaptic contacts (Bading et al., 1995; Hardingham et al., 2001b). Action potential bursting in hippocampal neurons was induced at day *in vitro* 10 by supplementing the medium with the $GABA_A$ receptor antagonist Bicuculline [50µM] (Alexis) for 1h to 16h (Arnold et al., 2005). For the pharmacological inhibitor experiments, neurons were treated for 2h to 4h with Bicuculline either with or without a 45min pretreatment with the pharmacological inhibitors MK 801 [10µM] (Sigma), PD 98059 [20µM] (Calbiochem), SB 203580 [10µM] (Calbiochem), or

KN 62 [5µM] (Calbiochem), FK 506 [1µM] (Axxora) and Cyclosporin A [1µM] (Sigma-Aldrich), and Anisomycin [20µg/ml] (Applichem).

Cells were harvested in RLT Lysis Buffer (Qiagen) and RNA was isolated using RNeasy Mini Kit (Qiagen) according to manufacturer's instructions, with additional on-column DNase digestion during RNA purification. cDNA was synthesized from 1µg to 2µg of total RNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to manufacturer's instructions.

RT-qPCR (reverse transcriptase quantitative reverse transcriptase PCR) was done on an ABI7300 thermal cycler using universal qPCR master mix with TaqMan Gene Expression Assays (Applied Biosystems) for the following genes: *Gusb* (Mm00446953_m1), *c-fos* (Mm00487425_m1), *Atf3* (Mm00476032_m1), *Lrrtm1* (Mm00551337_g1) and *Lrrtm2* (Mm00997210_g1). The expression levels of the target genes were normalized to the relative ratio of the expression of the housekeeping gene *Gusb*. For analyses of statistical significance *t* tests (two-sample assuming equal variances) were performed. Data for *Lrrtm1* and *Lrrtm2* represent mean values ± SEM (standard error of the mean) from three independent experiments. Data for *c-fos* and *Atf3* were log-transformed and autoscaled; means and standard deviations (SDs) were calculated and *t* tests for analyses of statistical significance were performed (two-sample assuming equal variances) (Pruunsild et al., 2011). For graphical representation, the data were back-transformed to the original scale. Error bars represent upper and lower limits back-transformed as mean ± SD. Data from three independent experiments are shown.

Calcium imaging

Imaging of Bicuculline-induced calcium signals was done with mouse hippocampal neurons plated on PDL/LA-coated cover slips. After a culturing period of 10 to 12 days *in vitro* neurons were loaded with Fluo-3 [3.8µM] (Invitrogen) for 45 min in CO_2-independent Salt Glucose Glycine solution (SGG^{ind}; [140.1mM NaCl, 5.3mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$, 10mM HEPES, 1mM glycine, 30mM glucose, and 0.5mM sodium pyruvate]) (Bading et al., 1993). Following the incubation period, cells were washed 5x with SGG^{ind} and kept in SGG^{ind} for 45min in the presence of KN 62 [5µM]. Action

potential bursting was induced by Bicuculline [50µM]. Calcium signals were detected using a Leica SP2 confocal microscope and imaging software (Leica). Calcium concentrations were expressed as a function of the Fluo-3 fluorescence $[(F-F_{min})/(F_{max}-F)]$ using Ionomycin [50µM] (Sigma) and saturated $MnCl_2$ solution to obtain F_{max} and F_{min}.

Immunodetection

For immunoblot analysis, untreated or pharmaceutically treated cells were harvested in sample buffer (9% SDS, 187.5mM Tris, 30% glycerol, 10mM DTT, 33mM EDTA, bromphenol blue) and stored at –20°C until usage. Gel electrophoresis and immunoblotting of protein samples were performed with a wet blotter system using two-layer acrylamid gels (3.75%, 12%) according to standard western blot procedures. For visualization of immunoblots, HRP-based secondary antibodies (Sigma) were used followed by chemiluminescent detection on film (GE Healthcare Limited). Antibodies (ab) to the following proteins were used: α-MeCP2 (rabbit polyclonal ab, 1:1000; Millipore), α-phosphoMeCP2 (rabbit polyclonal ab, 1:1000; a kind gift from M.E. Greenberg (Zhou et al., 2006)), α-Tubulin (mouse monoclonal ab, 1:400000; Sigma). Immunoblots were quantified using ImageJ Software; phosphoMeCP2 signal was normalized to the signal of total amount of MeCP2. The ratios were set relative to the signal of day *in vitro* 3 of the first experiment. Three independent experiments were performed. Data represent mean values ± SEM.

Luciferase assay

Cloning. A 356bp long sequence of the *Lrrtm2* promoter region was amplified from mouse genomic DNA with the PyroStart Fast PCR Master Mix (Fermentas) using the following primers: sense, 5'–CTCGAGAGCTCTCACACGCATTAGAA–3';

antisense, 5'–AGATCTCAGCATGAGTGCATTTACTG–3'

and cloned into pGL4.10[*luc2*] (Promega) in front of the firefly luciferase coding sequence (*Lrrtm2*^{WT}-luc). The CRE sites were mutated from CGTCA to CcaCA and from

TGACGTCA to TGtgGTCA (*Lrrtm2*$^{\Delta CRE}$-luc) by overlap extension PCR using Phusion High Fidelity DNA Polymerase (New England Biolabs) and the following primers:

sense, 5'–ACAAAAGACACcaCACCCGGTGtgGTCAGC–3';

antisense, 5'–GCTGACcaCACCGGGTGtgGTGTCTTTTGT–3'.

The accuracy of the promoter regions was verified by sequencing.

Cell culture, transfection, and pharmacological treatments. Rat hippocampal neurons from newborn Sprague Dawley rats (Charlers River) were plated on PDL/LA-coated culture dishes at a density of 0.25 hippocampi per 1ml NBA containing 1% rat serum and B27 (Invitrogen). Transfection was done on day *in vitro* 10 with *Lrrtm2*^{WT}-luc and *Lrrtm2*$^{\Delta CRE}$-luc, respectively, using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. pGL4.29[*luc2P*/CRE/Hygro] (Promega), a plasmid containing a CRE site as reporter gene-driving element, was included as positive control. For normalization of the luciferase assays, pGL4.83[*hRlucP*/Puro] (Promega) containing the promoter of human EF1α (elongation factor-1α; a kind gift from P. Pruunsild and M. Sepp (Sepp et al., 2012)) was used at a ratio of 10:1 (promoter : normalizer). Transfection was performed using 1µg plasmid DNA plus 1µl Lipofectamin 2000 in a final volume of 250µl NBA medium. The neurons were pretreated with APV (2-amino-5-phosphonovaleric acid) [20µM] (Biotrend) over night and stimulated on day *in vitro* 11 with Bicuculline [50µM] and 4-AP (4-aminopyridine) [250µM] (Sigma) for 8h.

Measurement. The luciferase assay was performed using the Dual-Glo Luciferase Assay System (Promega) according to manufacturer's instructions. Chemiluminescence was measured by GloMax Luminometer and Software (Promega and Turner Biosystems). Induction values were considered when the positive control reached $\geq$ 100-fold relative luciferase units. For presentation of the relative luciferase activity data, the background signals from untransfected neurons were subtracted from signals obtained from transfected cells. For analyses of statistical significance *t* tests were performed (two-sample assuming equal variances). Data represent mean values ± SEM from four independent experiments, each performed in triplicates.

AAV vector cloning, virus production and infection

Cloning. The wild-type coding sequence of *MeCP2* was amplified from a template plasmid (a kind gift from M.E. Greenberg (Zhou et al., 2006)) with the PyroStart Fast PCR Master Mix (Fermentas) using the following primers:

sense: 5'–CGCGGATCCATGGTAGCTGGGATGTTAGGGCTCA–3',

antisense: 5'–CTAGCTAGCGCTAACTCTCTCGGTCACGG–3',

and cloned into a WPRE- and polyA-sequence-containing rAAV (recombinant adeno-associated virus) plasmid under the control of a 1.3kbp fragment of the mouse CaMKII promoter (a kind gift from P. Seeburg) (*MeCP2*^{WT}). A non-phosphorylatable form of MeCP2 at serine 421 was cloned by creating a serine-to-alanine exchange (TCA>gCA; *MeCP2*^{S421A}) through overlap extension PCR using Phusion High Fidelity DNA Polymerase (New England Biolabs) and the following primers:

sense: 5'–CCTGAGAGCTCTGAGGACCCCATCAGCCCC–3',

antisense: 5'–CTAGCTAGCGCTAACTCTCTCGGTCACGG–3';

overlap extension sense: 5'–ATGCCCCGAGGAGGCgCACTGGAAAGCGAT–3';

overlap extension antisense: 5'–ATCGCTTTCCAGTGcGCCTCCTCGGGGCAT–3'.

The expression vector for VP16-MeCP2 was cloned by inserting wild-type *MeCP2* into a rAAV plasmid containing a CMV/CBA promoter (Klugmann et al., 2005) and the coding sequence of VP16 (*VP16-MeCP2*).

The shRNA (short hairpin RNA) sequences for *Lrrtm1* and *Lrrtm2* were generated using Dharmacon siDesignCentre (Thermo Scientific). The oligomers were annealed and cloned into a rAAV expression vector under the control of the U6 promoter. As reporter gene, the vector carried the coding sequence for mCherry under the control of the CaMKII promoter. For *Lrrtm1,* three shRNA sequences were designed and cloned. For *Lrrtm2,* two sequences were designed and a third was taken from the literature (de Wit et al., 2009). The following sequences were cloned:

shLrrtm1-a: 5'–GCAGCAGCAAAGTGAGACA–3'

shLrrtm1-b: 5'–GGACACGAATGGCAGGCGT–3'

shLrrtm1-c: 5'–ACTCCAAGGTGGCTTCGAT–3'

shLrrtm2-a: 5'–TGCTATTCTACTGCGACTC–3' (de Wit et al., 2009)

shLrrtm2-b: 5'–GGGCACAGAAAACAGGAAA–3'

shLrrtm2-c: 5'–GAACTAACAGCTGCGGGAA–3'

In order to clone expression vectors for Lrrtm1 and Lrrtm2 (rAAV-*Lrrtm1* and rAAV-*Lrrtm2*), the coding sequence for *Lrrtm1* and *Lrrtm2* was generated by PCR using Phusion High Fidelity DNA Polymerase (New England Biolabs) with cDNA as template-DNA and the following primers:

Lrrtm1, sense: 5'–CGCGGATCCATGGATTTCCTGCTACTCGG–3'

Lrrtm1, antisense: 5'–CTAGCTAGCCACCTCGCATTCCCTCGCAG–3'

Lrrtm2, sense: 5'–CGCGGATCCATGGGCTTACATTTCAAGTG–3'

Lrrtm2, antisense: 5'–CTAGCTAGCTACTTCACATTCTTTGTATG–3'

The PCR product was cloned into a rAAV expression vector under the control of a CMV/CBA promoter.

For the Lrrtm1 rescue experiment, three different rAAV plasmids were constructed: rAAV-*unc*-Lrrtm1 (unc, universal control), rAAV-*shLrrtm1-mCherry*, and rAAV-*shLrrtm1*-Lrrtm1. A rAAV plasmid containing two expression cassettes for *unc* and *mCherry* controlled by a U6 promoter (unc) and a CaMKII promoter (mCherry), respectively, was obtained from H. E. Freitag. To create rAA-*shLrrtm1-mCherry*, the *shLrrtm1*-a sequence was inserted downstream of the U6 promoter in place of the *unc* sequence. To obtain rAAV-*unc*-Lrrtm1, the coding sequence for *Lrrtm1* was generated by PCR using Phusion High Fidelity DNA Polymerase (New England Biolabs) with cDNA as template-DNA and the following primers:

Lrrtm1, sense: 5'–CCGAGTACCGGTATGGATTTCCTGCTACTCGG–3'

Lrrtm1, antisense: 5'–CCGAGTGATATCCACCTCGCATTCCCTCGCAG–3'

The PCR product was cloned into rAAV-*unc-mCherry* downstream of the CaMKII promoter in place of the *mCherry* sequence. To create rAAV-*shLrrtm1*-Lrrtm1, the *shLrrtm1*-a sequence was inserted into rAAV-*unc*-Lrrtm1 downstream of the U6 promoter in place of the *unc* sequence. The integrity of the plasmids was verified by sequencing.

Virus production. The procedure of AAV production has been described previously (During et al., 2003; Hauck et al., 2003). In brief, human embryonic kidney 293 cells were transfected with the rAAV expression plasmid and the adeno helper plasmids pFδ6 (179), pH21 (180) for AAV capsid 1 protein expression, and pRV1 (181) for AAV capsid 2 protein expression (Hauck et al., 2003; Klugmann et al., 2005) by standard calcium

phosphate transfection. 72h after transfection, cells were harvested and the virus was purified using HiTrap Heparin Columns (GE Healthcare). The following rAAVs were produced: rAAV-*mCherry-NLS* (rAAV-*mCherry-NLS* plasmid was a kind gift from H. E. Freitag), rAAV-*MeCP2*^{WT}, rAAV-*MeCP2*^{S421A}, rAAV-*VP16-MeCP2,* rAAV-*shLrrtm1* (a-c), rAAV-*shLrrtm2* (a-c), rAAV-*Lrrtm1*, rAAV-*Lrrtm2*, and rAAV-*shLrrtm1*-Lrrtm1.

Infection. Cultured mouse hippocampal neurons were infected on day *in vitro* 3 to 7 by addition of the virus to the medium. The following rAAVs were used: rAAV-*mCherry-NLS*, rAAV-*CaMBP4-mCherry* (a kind gift from D. Lau), rAAV-*E1A*, rAAV-*E1AΔCR1* (both a kind gift from D. Mauceri (Mauceri et al., 2011)), rAAV-*MeCP2*^{WT}, rAAV-*MeCP2*^{S421A}, rAAV-*VP16-MeCP2*, rAAV-*shLrrtm1*, rAAV-*shLrrtm2*, rAAV-*Lrrtm1*, rAAV-*Lrrtm2*, and rAAV-*shLrrtm1*-Lrrtm1.

MeCP2 data represent mean values ± SEM from three independent experiments. Data for CaMBP4 and E1A were log-transformed and autoscaled; means and standard deviations were calculated and *t* tests for analyses of statistical significance were performed (two-sample assuming equal variances) (Pruunsild et al., 2011). For graphical representation, the data were back-transformed to the original scale. Error bars represent upper and lower limits back-transformed as mean ± SD from three independent experiments.

Chromatin immunoprecipitation

Chromatin immunoprecipitation. The chromatin immunoprecipitation was performed using Magna ChIP Chromatin Immunoprecipitation Kit (Millipore) according to manufacturer's instructions. In brief, 3 dishes (diameter 6cm) of cultured mouse hippocampal neurons, day *in vitro* 11 to 12, were treated with Bicuculline [50µM] for 0.5h to 4h and then fixed by addition of freshly prepared paraformaldehyde [1%]. Cells were harvested, and lysed in a total volume of 300µl Nuclear Lysis Buffer (Millipore). Chromatin was sheared to fragments of 200bp to 1000bp by applying 8 pulses of 5sec duration and 20% power output using a Branson Digital Sonifier. 50µl of sonicated DNA per immunoprecipitation were incubated with 2µg α-CREB or 8µg α-c-Jun antibody and magnetic protein A/G beads (Millipore) over night at 4°C. Protein/DNA complexes were eluted in 100µl Elution

Buffer (Millipore) and DNA was purified using QIAquick PCR Purification Kit (Qiagen). The following antibodies were used: α-CREB (rabbit monoclonal ab, Cell Signaling Technology), α-c-Jun (rabbit polyclonal ab, Santa Cruy Biotechnology), rabbit IgG (Santa Cruz Biotechnology).

qPCR analysis. Purified DNA was diluted 1:10 for analysis. The immunoprecipitated DNA was analysed by quantitative PCR using Power SYBR Green PCR Master Mix (Applied Biosystems) and the following promoter-specific primers with an annealing temperature of 55°C:

p*c-fos* sense: 5'–AGATGTATGCCAAGACGGGGG–3',

antisense: 5'–CAGTCGCGGTTGGAGTAGTAG–3';

p*Mef2c* sense: 5'–CACTTGAGCACACGCGTACA–3',

antisense: 5'–ACCCACACAGAACCTTCAAAGTC–3';

p*Ccrn4l* sense: 5'–CGGAACGCCTCTCTAACGAA–3',

antisense: 5'–GGACCGTCTGGATCAGTGAC–3';

p*Jun* sense: 5'–GGAGCATTACCTCATCCCGT–3',

antisense: 5'–ATTGGCTTGCGTCGTTCTCA–3'

p*Lrrtm1* sense: 5'–TCGAGCCCCGAGTTTGGAGTT–3',

antisense: 5'–TGCTTCTCGCCTTCCTGCCT–3';

p*Lrrtm2* sense: 5'–CGCCCCTGACACTGTTACAA–3',

antisense: 5'–CCGAGAAACGGCACAAGAAT–3'.

Four independent experiments were performed (α-c-Jun: two), and each sample was measured in triplicate with primers detecting the gene-specific regions. The amount of immunoprecipitated DNA is presented as 'percent of input' and 'fold enrichment over IgG', respectively. Statistical significance was assessed by *t* test; the data represent mean values ± SEM.

Morphological analyses

For the analyses of spine density, cultured mouse hippocampal neurons grown on PDL/LA-coated cover-slips were infected with rAAV-*shLrrtm2* or a control virus, rAAV-

shScramble at day *in vitro* 7. At day in vitro 10-11 neurons were transfected with rAAV-*EGFP* to visualize individual neurons. One to two days after transfection, neurons were fixed and counterstained with Hoechst 33258. Single neurons were imaged using a Leica SP2 confocal microscope and imaging software (Leica). A z-stack projection of on average 10 sections was created. In each independent experiment, at least four neurons were imaged per condition. The spines of at least two 20µm sections of a secondary dendrite were counted manually for each neuron using ImageJ software (ImageJ 1.43u, Wayne Rasband, NIH). All analyses were performed blind.

AMPA receptor trafficking

Life cell imaging. Imaging of AMPA receptor trafficking was done with mouse hippocampal neurons plated on PDL/LA-coated cover slips. After a culturing period of 10 to 12 days *in vitro* neurons were transfected with pCl-SEP-GluR2 (addgene). Two days after transfection the cells were kept over night in no-glycine medium (NoG; [114mM NaCl, 26mM NaHCO3, 5.3mM KCl, 2mM CaCl2, 10mM HEPES, 30mM glucose, 0.5mM sodium pyruvate, 1M MgCl2], supplemented with Minimum Essential Medium (MEM, with Earle's salt, without L-glutamine) (Invitrogen), Insulin-transferrin-sodium selenite media supplement [6.3µg/ml-5.7µg/ml-7.5µg/ml] (Sigma), and Penicillin/Streptomycin solution [1:200] (Sigma)). The following day the cells were placed in a perfusion chamber and stimulated for 3min with chemical LTP using conditioned medium (CM; 114mM NaCl, 26mM NaHCO3, 5.3mM KCl, 3mM CaCl2, 10mM HEPES, 30mM glucose, 0.5mM sodium pyruvate, 200µM glycine, and 50µM Bicuculline) at room temperature. SEP-GluR/pHluorin signals were detected using a Leica SP2 confocal microscope and imaging software (Leica) and are plotted as raw fluorescence signal from dendritic regions of interest (ROIs) of transfected neurons over time. In addition, a second experimental setup was used with cultured hippocampal neurons plated on coverslips secured by a platinum ring in a perfusion chamber. The setup included heated in-line perfusion at 37°C. The extracellular solution was artificial cerebrospinal fluid (ACSF; [125mM NaCl; 3.5 KCl,;, 1.3mM $MgCl_2$, 1.2mM NaH_2PO_4, 2.4mM $CaCl_2$, 10mM glucose, 26mM $NaHCO_3$, gassed with 95% O_2 and 5% CO_2]). Neurons were viewed on a wide field

upright microscope (BX51WI, Olympus) equipped with a digital camera (sCMOS, Andor, BFi OPTiLAS) connected to a computer monitor through a PC interface using Andor IQ2 software.

Live cell staining. Prior to stimulation, the cells were kept over night in no-glycine medium (NoG; [114mM NaCl, 26mM $NaHCO_3$, 5.3mM KCl, 2mM CaCl2, 10mM HEPES, 30mM glucose, 0.5mM sodium pyruvate, 1M $MgCl_2$], supplemented with Minimum Essential Medium (MEM, with Earle's salt, without L-glutamine) (Invitrogen), Insulin-transferrin-sodium selenite media supplement [6.3µg/ml-5.7µg/ml-7.5µg/ml] (Sigma), and Penicillin/Streptomycin solution [1:200] (Sigma)). To cover existing AMPAs, neurons were incubated with anti-GluA1 antibody (oncogene, 1:5) at 4°C for 45min, followed by incubation with a cold secondary antibody at 4°C for 45min. Cells were then either left untreated or stimulated for 3min with conditioned medium (CM; 114mM NaCl, 26mM $NaHCO_3$, 5.3mM KCl, 3mM $CaCl_2$, 10mM HEPES, 30mM glucose, 0.5mM sodium pyruvate, 200µM glycine, and 50µM Bicuculline) at room temperature. Following the stimulation with CM, neurons were kept for further 15min in NoG at room temperature. After the procedure, all cells were fixed in 4% sucrose/ 4% paraformaldehyde solution and incubated with the same primary antibody as before, anti-GluA1 ab, followed by incubation with an DyLight488- or Cy3-conjugated secondary antibody (both Dianova) in order to visualize the newly externalized AMPA receptors.

Fixed cell staining. Mouse hippocampal cultures were transfected with pCL-SEP-GluR2 (addgene) on day in vitro 10-12. One to two days after transfection, neurons were either left untreated or stimulated with 50µM Bicuculline for 10min. Neurons were then fixed in 4% sucrose/ 4% paraformaldehyde solution for 15min. Following washing with PBS (3x), cells were blocked in blocking solution (10% NGS, 2% BSA in 0.1% PBST). Without washing, cells were incubated with the primary antibody (rabbit-anti-GFP (Invitrogen), 1:500 in blocking solution) over night at 4°C. Subsequently the cells were washed with PBS (5x) for 1h and then incubated with a secondary antibody (A) (anti-rabbit-Cy3 (Dianova), 1:500 in PBS) for 45min at room temperature. Afterwards the cells were washed with PBS (5x) for 1h and then permeabilized with 0.1% TritonX 100 for 45min at room temperature. Following washing with PBS (2x), cells were blocked in blocking solution for 45min at room temperature. Subsequently the cells were again incubated with the same primary antibody (rabbit-anti-GFP, 1:500 in blocking solution) over night at 4^0C and then washed with PBS (4-5x) for 1h. Afterwards the cells were incubated with a secondary antibody (B) (anti-rabbit-DyLight488 (Dianova), 1:500 in PBS) for 45min at

room temperature. The cells were then washed once with PBS, incubated in Hoechst 33258 (Sigma, 1:10000) for 3min, washed again with PBS (4x) and mounted in Moviol.

Microelectrode array recordings

Except for virus production, this method was performed by H. E. Freitag. Hippocampal neurons from new-born Sprague-Dawley rats were plated onto MEA dishes containing a grid of 60 planar electrodes (Multi Channel Systems). Recordings were acquired with a MEA-60 amplifier board (10Hz–35 kHz, gain 1200, sampling frequency 20 kHz, Multi Channel Systems). The cultures were infected on day *in vitro* 4 with rAAV-*MeCP2*^{WT}, rAAV-*MeCP2*^{S421A}, rAAV-*shLrrtm1*, rAAV-*shLrrtm2*, rAAV-*Lrrtm1*, rAAV-*Lrrtm2*, and rAAV-*shLrrtm1*-Lrrtm1, respectively. Recordings were started after a culturing period of 7 days. Spikes were detected with the integrated spike detector of the MC Rack software (Multi Channel Systems). Burst analysis was done with Neuroexplorer (NEX Technologies). All results are given as mean ± S.E.M.

Results

Activity-dependent expression of *Lrrtm1* and *Lrrtm2*

The Lrrtm family has recently been identified as a group of proteins that is able to induce synaptic differentiation (Linhoff et al., 2009). Their mRNA levels were shown to be developmentally regulated, with increasing expression during embryogenesis and a peak level at the day of birth that persists into adulthood (Lauren et al., 2003). As many mechanisms that are important during development might occur in a similar fashion during neuronal activity and synaptic plasticity, I investigated whether *Lrrtm1* and *Lrrtm2*, the two most studied members of the Lrrtm family, are regulated by neuronal activity. In order to study their activity-induced expression, a network of cultured hippocampal neurons was exposed to the $GABA_A$ receptor antagonist Bicuculline. GABAergic interneurons, which represent about 10% of the neuronal population, impose a tonic inhibition onto the neuronal network (Arnold et al., 2005). Removal of $GABA_A$ergic inhibition with Bicuculline leads to action potential bursting (Arnold et al., 2005), which stimulates calcium entry through synaptic NMDA receptors, induces nuclear calcium-dependent transcription, and activates a variety of gene programmes (Hardingham et al., 2001b; Lee et al., 2005; Papadia et al., 2005; Zhang et al., 2009). A time course analysis using Bicuculline treatment to induce action potential bursting between 1h and 16h revealed a peak induction of *Lrrtm1* and *Lrrtm2* mRNA at 4h and 2h, respectively (Figure 1A). The induction was detected using quantitative reverse transcriptase PCR and showed an about two fold increase in *Lrrtm1* and *Lrrtm2* mRNA levels. Similar to *c-fos*, a classic neuronal activity- and calcium-regulated gene (Bading et al., 1993; Curran and Morgan, 1995), this induction of *Lrrtm1* and *Lrrtm2* by synaptic activity could be blocked by application of MK 801, a selective non-competitive NMDA receptor antagonist that prevents the Bicuculline -induced calcium entry into the neuron (Figure 1B). Further, *Lrrtm1* and *Lrrtm2* are classic immediate-early genes, since their induction is independent of on-going protein translation, which could be shown by inducing neuronal activity in the presence of Anisomycin, a protein synthesis inhibitor (Figure 1C). The superinduction of *Lrrtm1* and

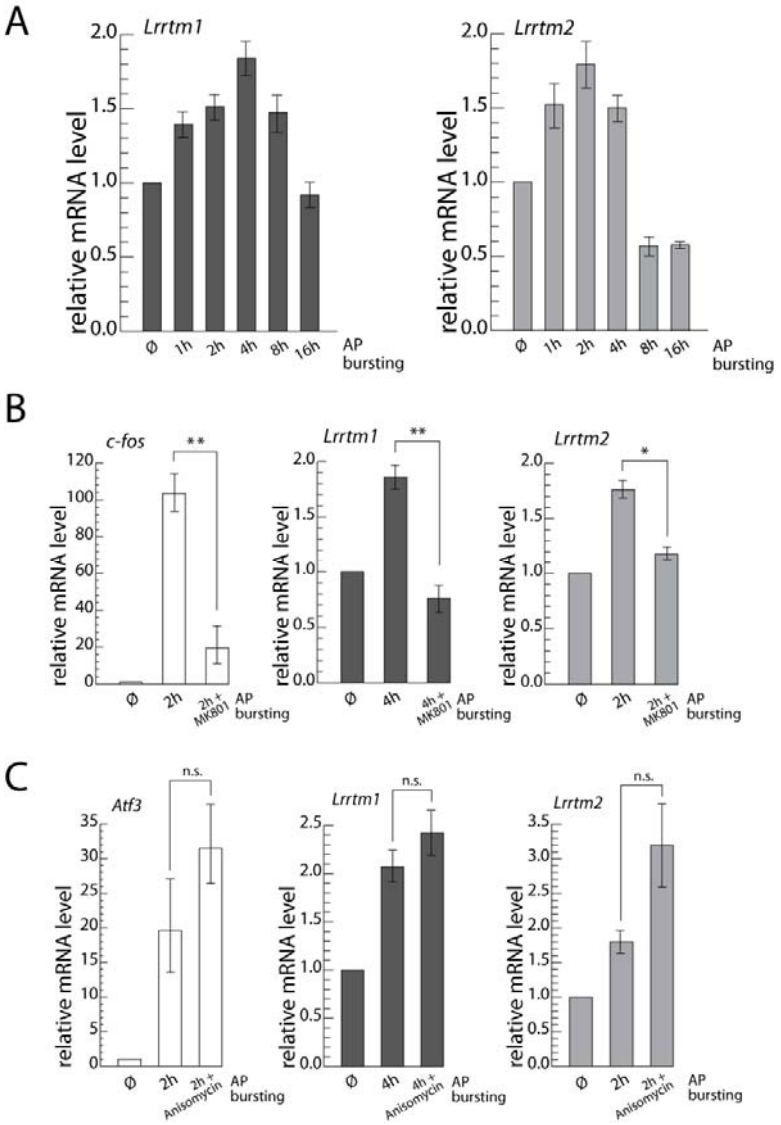

Figure 1. Activty-dependent regulation of *Lrrtm1* and *Lrrtm2* requires the activation of NMDA receptors and follows an immediate-early expression pattern.

A, Primary mouse hippocampal neurons were used for studying *Lrrtm1* and *Lrrtm2* regulation in response to neuronal activity modelled by Bicuculline stimulation, a $GABA_A$ receptor antagonist that removes inhibitory synaptic activity, giving rise to action potential bursting. Neurons (day *in vitro* 10) were treated with 50µM Bicuculline for the time indicated and endogenous *Lrrtm1* and *Lrrtm2* mRNA levels were measured by RT-qPCR using gene-specific primers. mRNA levels are shown as fold induced levels of *Lrrtm1* and *Lrrtm2* over levels in untreated neurons. **B**, **C**, Neurons as in A were used for analysis of *Lrrtm1* and *Lrrtm2* mRNA expression after treatment with Bicuculline for the time indicated in the presence or absence of either 10µM MK 801, a selective non-competitive NMDA receptor antagonist that prevents the Bicuculline-induced calcium entry into the neuron (**B**), or Anisomycin (20µg/ml), a protein synthesis inhibitor (**C**). *Lrrtm1* and *Lrrtm2* mRNA levels were measured by RT-qPCR using gene-specific primers and are shown as fold induced levels over levels in untreated neurons. The data in A-C were obtained from three independent experiments with duplicate measurements and normalized to *Gusb* expression. *Lrrtm1* and *Lrrtm2* data are mean values ± SEM, c-*fos* and *Atf3* data are mean values ± SD. Statistical significance was assessed by *t* test (* $p < 0.05$; ** $p < 0.005$). Ø, unstimulated; AP, action potential; n.s., not significant.

Lrrtm2 expression in the presence of Anisomycin is a phenomenon regularly detected under the usage of protein synthesis inhibitors (Mahadevan and Edwards, 1991) and was also apparent for *Atf3*, a control gene that was analysed in parallel. Collectively, these data show that *Lrrtm1* and *Lrrtm2* are classic immediate-early genes that are induced and tightly controlled by synaptic activity through an NMDA receptor-dependent mechanism.

Signalling pathways involved in activity-induced expression of *Lrrtm1* and *Lrrtm2*

The four major neuronal calcium signalling pathways implicated in transcriptional regulation are controlled by CaM kinases, calcineurin, p38 MAP kinases, and MAPK/ERK. In order to identify the pathways that execute the signalling from the synapse to the nucleus in the regulation of *Lrrtm1* and *Lrrtm2*, I used pharmacological inhibitors of these signalling pathways. I found that neither inhibition of MAPK/ERK kinases, p38 MAP kinases nor calcineurin using PD 98059, SB 203580, or FK 506 plus Cyclosporin A, respectively, compromised the observed increase in *Lrrtm1* and *Lrrtm2* expression after Bicuculline-induced action potential bursting (Figure 2). In contrast, blockade of the CaM kinases using KN 62 completely abolished the activity-induced increase in *Lrrtm1* and *Lrrtm2* mRNA levels (Figure 3). *Atf3*, a gene also controlled by neuronal activity, was analysed in parallel and served as positive control. In the promoter of *Atf3* are a CRE and a MRE site, which confer responsiveness to the transcription factors CREB and MEF2, respectively (Zhang et al., 2011). While CREB is mainly activated by ERK and CaM kinase IV (Chawla et al., 1998; Sgambato et al., 1998), MEF2 is controlled by p38 kinases (Mao et al., 1999), CaMK II (McKinsey et al., 2000; Linseman et al., 2003) and calcineurin, which enhances MEF2 DNA binding activity (Mao and Wiedmann, 1999). Consequently, *Atf3* expression in response to neuronal activity is compromised by inhibition of each of these four pathways (Figure 2, 3A). The blockade of CaM kinases has the most prominent effect, most likely due to the inhibition of both the CREB and the MEF pathway (Figure 3A).

Since there is a known inhibitory effect of KN 62 on voltage-gated calcium channels that

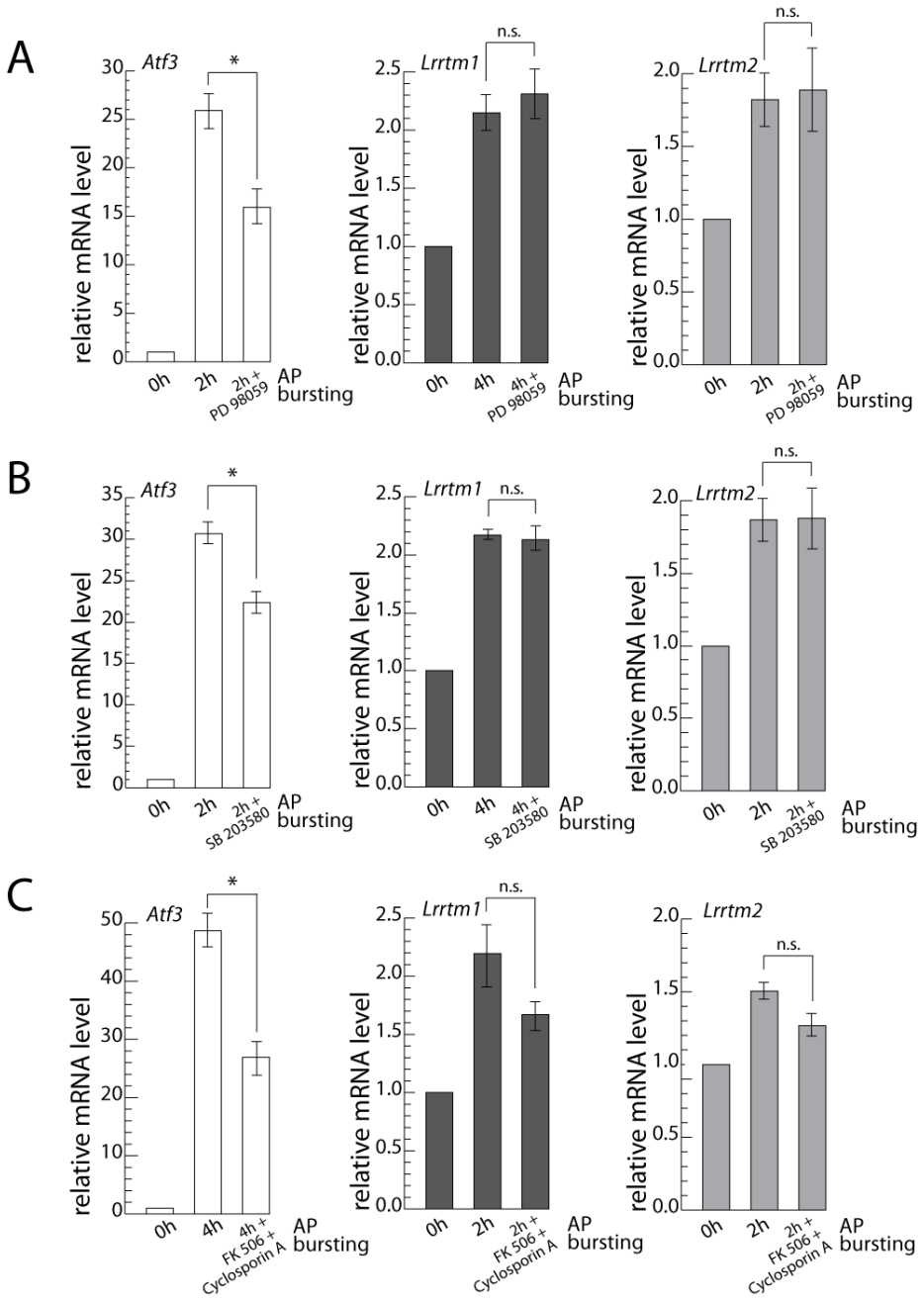

Figure 2. Activty-dependent regulation of *Lrrtm1* and *Lrrtm2* is independent of MEK1, p38 and CaM/cacineurin signalling.

A-**D**, Primary mouse hippocampal neurons were used for studying *Lrrtm1* and *Lrrtm2* regulation by activity-dependent intracellular signalling pathways. Action potential bursting was induced for the time indicated by stimulation with 50µM Bicuculline either in the presence or absence of the denoted inhibitors. mRNA expression of endogenous *Lrrtm1* and *Lrrtm2* was measured by RT-qPCR using gene-specific primers and mRNA levels are shown as fold induced levels of *Lrrtm1* and *Lrrtm2* over levels in untreated neurons. **A**, Inhibition of the MEK/ERK signalling pathway by the MEK1 inhibitor PD 980598 (20µM). **B**, Inhibition of p38 by SB 203580 (10µM). **C**, Inhibition of CaM/calcineurin signalling by the calcineurin inhibitors FK 506 (1µM) plus Cyclosporin A (1µM). **D**, Inhibition of the calcium/calmodulin-dependent kinase pathway by KN 62 (5µM). The data in A-D were obtained from three independent experiments with duplicate measurements and normalized to *Gusb* expression. *Lrrtm1* and *Lrrtm2* data are mean values ± SEM, *Atf3* data are mean values ± SD. Statistical significance was assessed by *t* test (* $p < 0.05$; ** $p < 0.005$). AP, action potential; n.s., not significant.

might affect the generation of action potential bursting (Sihra and Pearson, 1995; Gao et al., 2006), I performed calcium imaging experiments to ensure that KN 62 application did

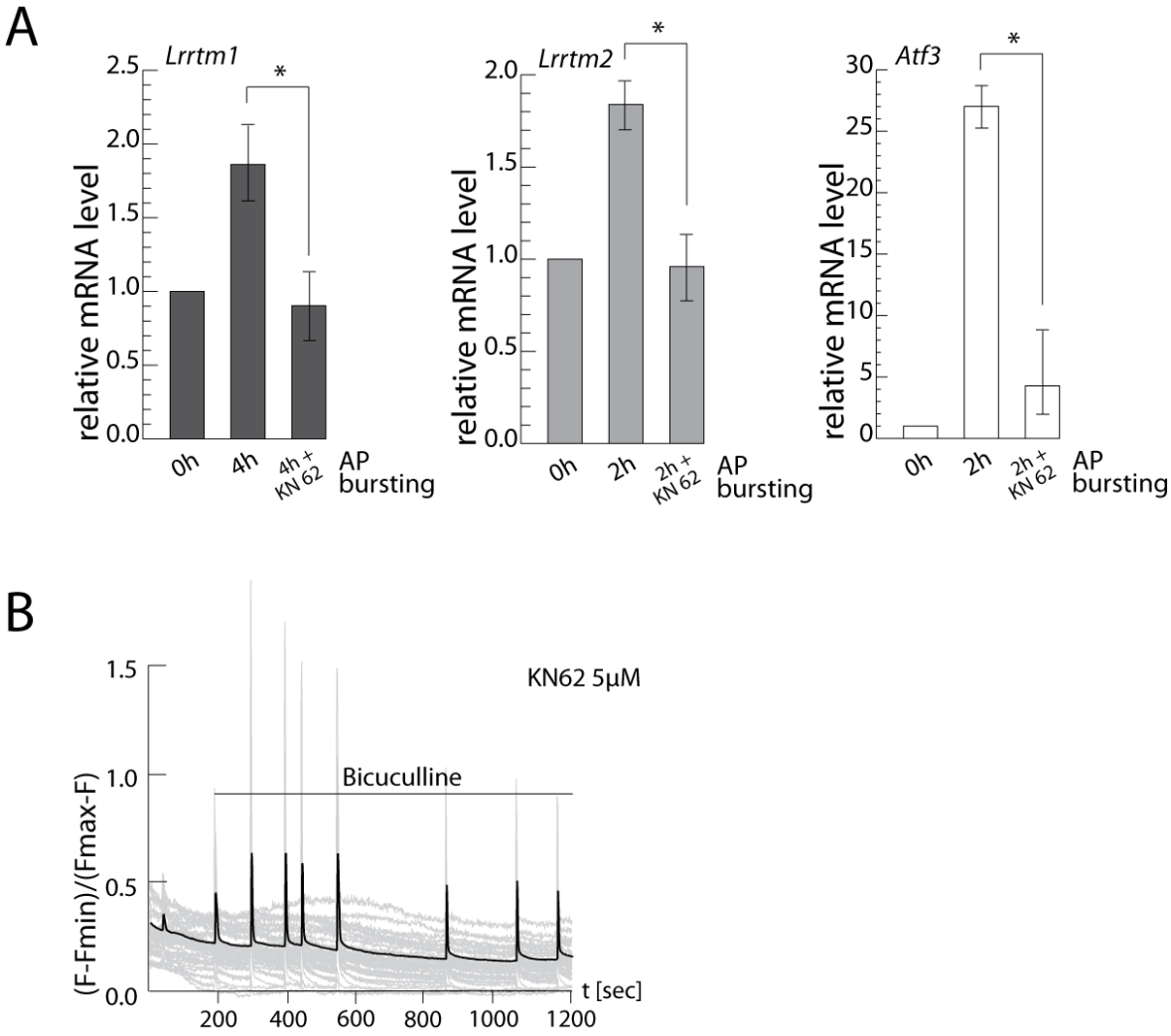

Figure 3. Activity-dependent regulation of *Lrrtm1* and *Lrrtm2* requires CaMKs signalling. A, Inhibition of the calcium/calmodulin-dependent kinase pathway by KN 62. Primary mouse hippocampal neurons were used for studying *Lrrtm1* and *Lrrtm2* regulation by CaMKs. Action potential bursting was induced for the time indicated by stimulation with 50µM Bicuculline either in the presence or absence of KN 62 (5µM). mRNA expression of endogenous *Lrrtm1* and *Lrrtm2* was measured by RT-qPCR using gene-specific primers and mRNA levels are shown as fold induced levels of *Lrrtm1* and *Lrrtm2* over levels in untreated neurons. Data were obtained from three independent experiments with duplicate measurements and normalized to *Gusb* expression. *Lrrtm1* and *Lrrtm2* data are mean values ± SEM. Statistical significance was assessed by *t* test (* $p < 0.05$). **B**, Neurons as in A were used to ensure unaltered bursting activity in the presence of 5µM KN 62. Neurons were loaded with the calcium indicator Fluo-3 and stimulated with Bicuculline at the indicated time point. Grey lines represent the measured Fluo-3 signal of individual cells; the mean signal is depicted as black line. Representative traces from three independent experiments are shown. AP, action potential.

not interfere with activity-induced calcium influx into the neuron. I found that in the hippocampal culture system, KN 62, when used at a concentration of 5µM, did not compromise Bicuculline-induced calcium transients (Figure 3B).

Taken together, the pharmacological experiments revealed that neither MAPK/ERK kinase, p38 MAPK, nor calcineurin are critically involved in regulating the Bicuculline-induced increase in *Lrrtm1* and *Lrrtm2* mRNA levels. However, synaptic activity-induced expression of both *Lrrtm1* and *Lrrtm2* is specifically regulated by a CaM kinase-dependent pathway.

Nuclear calcium signalling regulates the induction of *Lrrtm1* and *Lrrtm2*

The involvement of CaM kinases in the activity-dependent regulation of *Lrrtm1* and *Lrrtm2* directed my focus on the study of nuclear calcium signalling as a possible inducer of CaM kinases and subsequent *Lrrtm1* and *Lrrtm2* expression. Nuclear calcium is a known activator of nuclear-localized CaM kinases, such as CaMK II and CaMK IV,

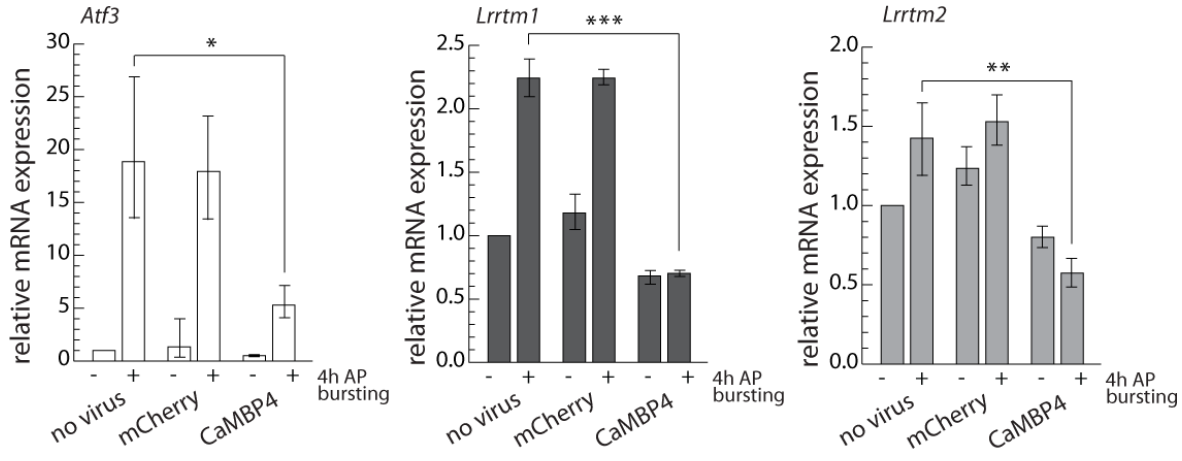

Figure 4. Nuclear calcium regulates *Lrrtm1* and *Lrrtm2* expression.
Primary mouse hippocampal neurons were used for studying *Lrrtm1* and *Lrrtm2* regulation by nuclear calcium signalling. Neurons (day *in vitro* 4) were infected with rAAV-*CaMBP4-mCherry*, or a control virus, rAAV-*mCherry*. Action potential bursting was induced by 50µM Bicuculline on day *in vitro* 10 for the time indicated. Expression levels of endogenous *Lrrtm1* and *Lrrtm2* mRNA were measured by RT-qPCR using gene-specific primers and are shown as fold induced levels of *Lrrtm1* and *Lrrtm2* over levels in untreated neurons. Data represent mean values ± SD from three independent experiments with duplicate measurements and normalized to *Gusb*. Statistical significance was assessed by *t* test (* $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$). AP, action potential.

both of which are important factors in mediating activity-induced genomic responses (Westphal et al., 1998; Impey et al., 2002; Wheeler et al., 2008; Zhang et al., 2009; Buchthal et al., 2012). In order to study the function of nuclear calcium signalling in the regulation of *Lrrtm1* and *Lrrtm2*, I used CaMBP4, a nuclear protein that contains four repeats of the M13 calmodulin binding peptide from the skeletal muscle myosin light chain kinase; it binds to and inactivates the nuclear calcium/CaM complex (Wang et al., 1995). CaMBP4 has previously been used to identify nuclear calcium-regulated genes that are important for neuroprotection and memory consolidation (Limback-Stokin et al., 2004; Zhang et al., 2009; Mauceri et al., 2011; Zhang et al., 2011). Primary mouse hippocampal neurons were infected with a recombinant adeno-associated virus (rAAV) containing an expression cassette for *CaMBP4* (rAAV-*CaMBP4-mCherry*). To induce action potential bursting, the cultures were exposed to Bicuculline, giving rise to periodically occurring action potential bursts, each of which is associated with an increase in the cytoplasmic and nuclear calcium concentration. In uninfected cultures this induced an about two-fold upregulation of *Lrrtm1* and *Lrrtm2* mRNA levels after 4h and 2h respectively. This upregulation was blocked in neurons infected with rAAV-*CaMBP4-mCherry*, but not in neurons infected with a control virus, rAAV-*mCherry* (Figure 4). This indicates that nuclear calcium signalling is required for synaptic activity-dependent regulation of *Lrrtm1* and *Lrrtm2*.

The role of CBP in *Lrrtm1* and *Lrrtm2* expression

In order to identify putative binding sites of transcription factors that are activated downstream of nuclear calcium signalling, I performed an on-line database search of a 2000bp-long upstream region of *Lrrtm1* and *Lrrtm2* genes using Transcription Element Search System (TESS; http://www.cbil.upenn.edu/cgi-bin/tess) to recognize transcription regulatory elements. The search retrieved a list of possible binding sites for numerous transcription factors in the promoter regions of *Lrrtm1* and *Lrrtm2*, including AP1 complex (activator protein 1), CREB, SP1 (specificity protein 1), USF (upstream stimulatory factor) and NFAT (nuclear factor of activated T-cells). CBP is a transcriptional co-activator that interacts with a variety of transcription factors (Shiama, 1997), including factors with

putative binding sites in the *Lrrtm1* and *Lrrtm2* promoters. Also, like the expression of *Lrrtm1* and *Lrrtm2*, nuclear calcium and CaM kinases control CBP activity (Chawla et al., 1998). Therefore I investigated the role of CBP in the regulation of *Lrrtm1* and *Lrrtm2* expression using a rAAV coding for the adenovirus protein E1A. E1A binds to CBP via its amino terminal-conserved region 1 (CR1) and disrupts CBP function (Arany et al., 1995; Bannister and Kouzarides, 1995). Mouse hippocampal neurons were infected with either rAAV-*E1A* or rAAV-*E1AΔCR1*, a control virus expressing E1A that lacks CR1 and fails to interact with CBP (Arany et al., 1995; Bannister and Kouzarides, 1995). To induce action potential bursting, the cultures were exposed to Bicuculline, which induced an about two-fold increase of *Lrrtm1* and *Lrrtm2* mRNA levels after 4h and 2h, respectively, in uninfected cultures. This increase was reduced in neurons infected with rAAV-*E1A*, but not in neurons infected with the control virus, rAAV-*E1AΔCR1* (Figure 5). This demonstrates that interference with CBP function compromises the activity-induced upregulation of *Lrrtm1* and *Lrrtm2* expression.

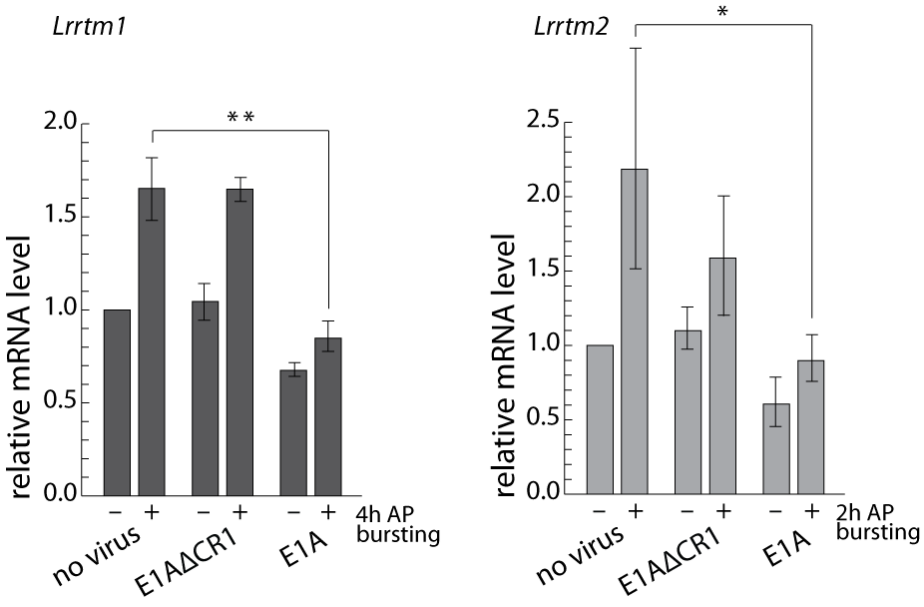

Figure 5. CBP regulates *Lrrtm1* and *Lrrtm2* expression.
Primary mouse hippocampal neurons were used for studying *Lrrtm1* and *Lrrtm2* regulation by CBP. Neurons (day *in vitro* 4) were infected with rAAV-*E1A*, or a control virus, rAAV-*E1AΔCR1*. Action potential bursting was induced by 50µM Bicuculline on day *in vitro* 10 for the time indicated. Expression levels of endogenous *Lrrtm1* and *Lrrtm2* mRNA were measured by RT-qPCR using gene-specific primers and are shown as fold induced levels of *Lrrtm1* and *Lrrtm2* over levels in untreated neurons. Data represent means ± SD from three independent experiments with duplicate measurements and are normalized to *Gusb*. Statistical significance was assessed by *t* test (* $p < 0.05$; ** $p < 0.005$). AP, action potential.

Lrrtm1 and *Lrrtm2* expression and CREB

An on-line database search of a 2000bp-long upstream region of *Lrrtm2* using Transcription Element Search System (TESS; http://www.cbil.upenn.edu/cgi-bin/tess) retrieved two CREs in the immediate vicinity of the transcription start site of *Lrrtm2*. Since CRE functions as a nuclear calcium response element (Hardingham et al., 1997), its presence in the *Lrrtm2* promoter might confer nuclear calcium responsiveness to this gene. The CRE is bound by CREB, a transcription factor which together with CBP forms a prototypical nuclear calcium-controlled transcription regulating complex (Chawla et al., 1998; Hardingham et al., 1999; Hardingham et al., 2001b). In a previous experiment I found that interference with CBP function impairs the activity-induced expression of *Lrrtm2* (see Figure 5). This impairment might be attributed to a disruption of CREB/CBP signalling; in this case, the activity-induced increase in *Lrrtm2* expression would depend on intact CRE sites in the promoter of *Lrrtm2*. In order to study the role of the CREs in the promoter of *Lrrtm2*, I constructed two *Lrrtm2* promoter-containing reporter plasmids. The wild-type reporter (p*Lrrtm2*^{WT}-*FLuc*) consists of a *firefly luciferase* (*FLuc*) reporter gene driven by a 356bp-long sequence of the mouse *Lrrtm2* promoter region harbouring a TATA box as well as a half and a full CRE site (Figure 6A, upper panel). Mutations were introduced into both CREs to generate a reporter construct that lacks the binding sites for CREB (p*Lrrtm2*$^{\Delta CRE}$-*FLuc*) (Figure 6A, lower panel). These constructs were transfected into hippocampal neurons and tested for their regulation by Bicuculline-induced neuronal activity. In preliminary experiments, no activity-induced increase in the reporter signal could be detected (data not shown). Yet, since the endogenous activity-dependent induction of *Lrrtm2* is comparatively low, one reason for this could be an insufficient sensitivity of the luciferase reporter system. In order to test this possibility, the neurons were cultured over night at low concentrations of APV, a selective NMDA receptor antagonist, followed by stimulation with Bicuculline in the presence of 4-AP, a reversible potassium channel blocker which increases the Bicuculline-induced bursting frequency in the neurons (Hardingham et al., 2002). This stimulation generated an induction that allowed monitoring of the increase in luciferase levels. In these experiments, I found that action potential bursting lead to a 1.5fold induction of the firefly reporter signal generated

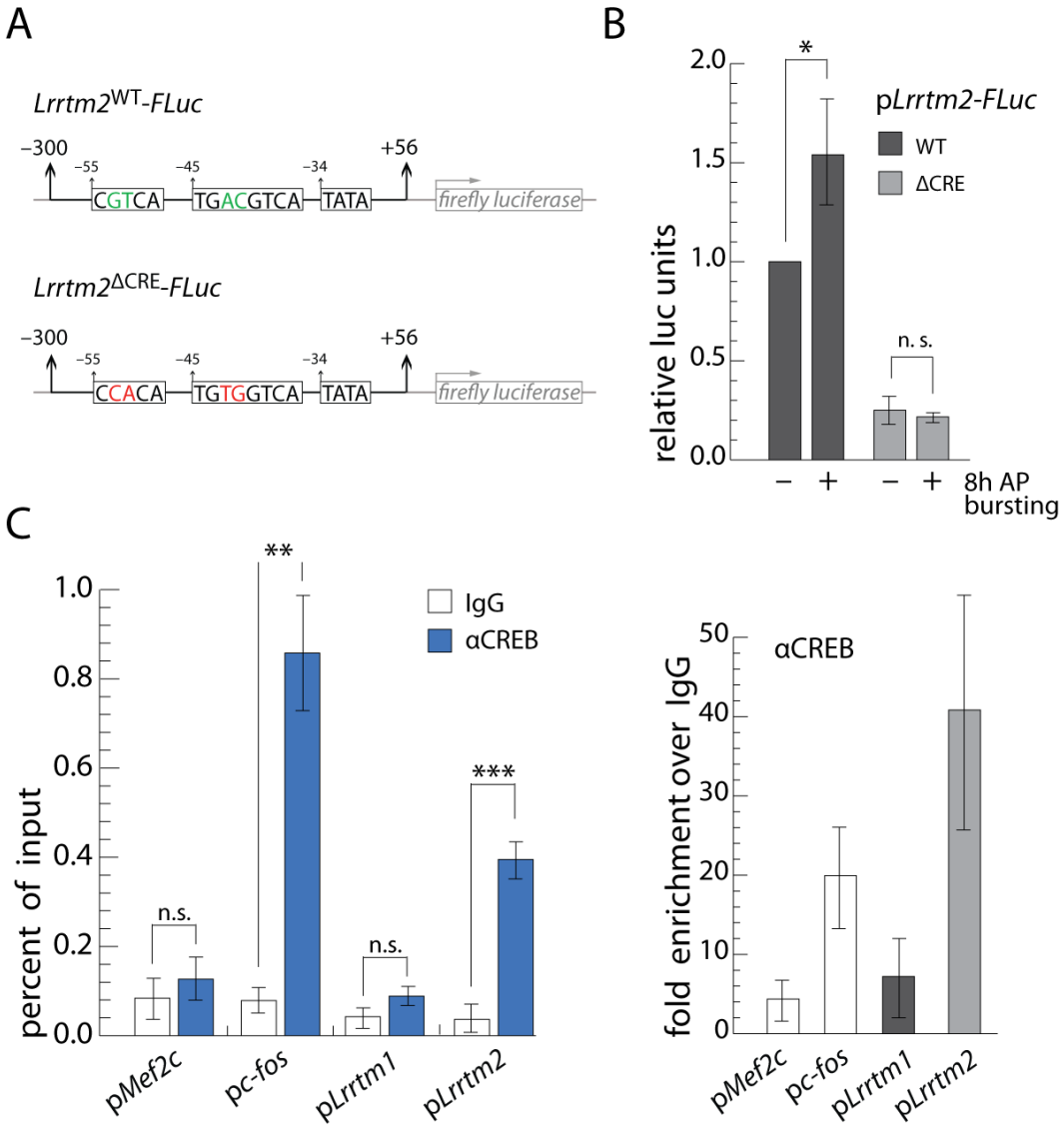

Figure 6. CREB binds to the CRE-containing promoter of *Lrrtm2*.
A, **B**, Primary rat hippocampal neurons were used for studying the CRE sites in the promoter of *Lrrtm2* by transfecting the neurons (day *in vitro* 10) with either wild-type (p*Lrrtm2*^{WT}*-FLuc*) or mutant (p*Lrrtm2*$^{\Delta CRE}$*-FLuc*) pGL4.10-based *firefly* luciferase (*FLuc*) reporter constructs (**A**) and an *EF1α* promoter-dependent humanized *Renilla* luciferase (*hRLuc*) construct. Following incubation with APV over night, action potential bursting was induced in transfected neurons on day *in vitro* 11 by Bicuculline (50µM) plus 4-AP (250µM) for 8h. Luciferase activities were measured and are represented as fold induced promoter activities over *Lrrtm2* wild-type promoter activities (**B**). Data represent mean values ± SEM from four independent experiments with triplicate measurements normalized to *hRluc* activities. Statistical significance was assessed by *t* test (* $p < 0.05$). **C**, *left panel*, Primary mouse hippocampal neurons were used to study CREB binding to the promoter of *Lrrtm1* and *Lrrtm2*. The binding was detected with anti-CREB ab in cell lysates from neurons treated with Bicuculline (50µM) for 30min. The data are represented for each indicated target gene as percent of input DNA determined by qPCR using promoter-specific primers. **C**, *right panel*, The same data as before are represented as fold enrichment of anti-CREB-immunoprecipitated DNA over IgG control for the respective target gene. The data represent mean values ± SEM from four independent experiments measured in triplicates and normalized to the levels of the respective target in the input DNA. Statistical significance was assessed by *t* test (** $p < 0.005$, *** $p < 0.0005$). AP, action potential; n.s., not significant.

by the wild-type promoter, p*Lrrtm2*^{WT}-*FLuc* (Figure 6B). This induction could not be observed with the firefly reporter driven by the mutant promoter, p*Lrrtm2*$^{\Delta CRE}$-*FLuc* (Figure 6B). Comparison of the reporter signals also revealed that the expression of the mutant promoter plasmid *Lrrtm2*$^{\Delta CRE}$-*FLuc* lead to a reduced basal firefly signal compared to wild-type, *Lrrtm2*^{WT}-*FLuc* (Figure 6B). This indicates that, under these conditions, the CRE sites are important elements in both the basal and activity-induced regulation of *Lrrtm2* expression.

In the next step, I performed chromatin immunoprecipitation experiments to investigate whether CREB binds to the genomic regions adjacent to the transcription start sites of *Lrrtm1* and *Lrrtm2*, respectively. Binding of endogenous CREB to the *Lrrtm1* and *Lrrtm2* regulatory regions was detected by immunoprecipitation of sheared chromatin from cultured hippocampal neurons using an antibody to CREB. The amount of immunoprecipitated DNA was measured by qPCR, with primers specific for the regulatory regions of *Lrrtm1* (p*Lrrtm1*) and *Lrrtm2* (p*Lrrtm2*). I observed a robust enrichment of the regulatory region of *Lrrtm2*, but not of *Lrrtm1* (Figure 6C). *c-fos*, a gene known to be regulated by CREB (Ofir et al., 1991; Bonni et al., 1995), was analysed in parallel and served as a positive control (p*c-fos*). As expected, the promoter of *c-fos* was enriched in the immunoprecipitated DNA (Figure 6C). In contrast, no significant enrichment could be measured for the promoter of *Mef2c*, an unrelated gene that was used as a negative control (p*Mef2c*; Figure 6C). These results show that CREB specifically binds to the analysed regulatory region of *Lrrtm2*, but not to the one of *Lrrtm1*.

The role of c-Jun in *Lrrtm1* and *Lrrtm2* expression

c-Jun is a transcription factor that interacts with CBP (Bannister et at., 1995) and according to the on-line database TESS it has putative binding sites in the promoter regions of *Lrrtm1* and *Lrrtm2*. c-Jun forms part of the AP-1 complex and is generally regarded as transcription factor activated in response to stress signals (Kyriakis et al., 1994). In addition it was shown that c-Jun initiates transcription as a result of an increase in the intracellular calcium concentration mediated by L-type voltage-gated calcium channels (Cruzalegui et al., 1999). The c-Jun-mediated transcription required CBP and CaM kinase

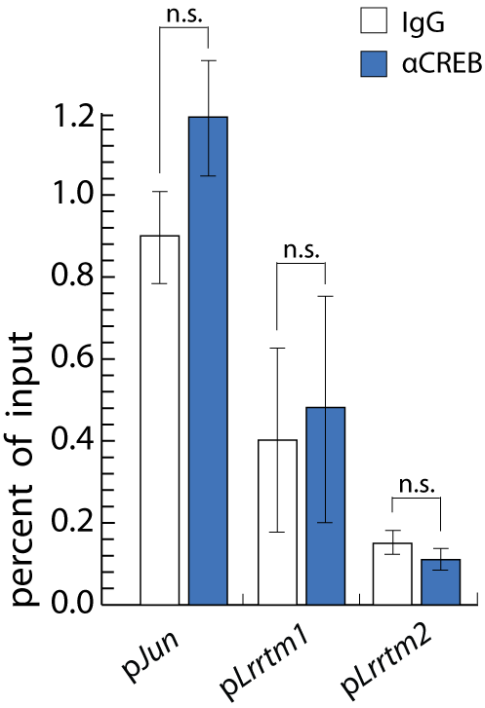

Figure 7. Chromatin immunoprecipitation using an antibody to c-Jun.
Primary mouse hippocampal neurons were used to study c-Jun binding to the promoter of *Lrrtm1* and *Lrrtm2*. The binding was detected with anti-c-Jun ab in cell lysates from neurons treated with Bicuculline (50µM) for four hours. The data are represented for each indicated target gene as percent of input DNA determined by qPCR using promoter-specific primers. The data represent mean values ± SEM from two independent experiments measured in triplicates and normalized to the levels of the respective target in the input DNA. Statistical significance was assessed by *t* test. n.s., not significant.

signalling (Cruzalegui et al., 1999), both of which were shown here to regulate the expression of *Lrrtm1* and *Lrrtm2*. Accordingly, there is the possibility that c-Jun mediates the activity-induced upregulation of *Lrrtm1* and *Lrrtm2* mRNA. In order to test this hypothesis, I performed chromatin immunoprecipitation to detect endogenous c-Jun binding to the regulatory regions of *Lrrtm1* and *Lrrtm2*, respectively (p*Lrrtm1* and p*Lrrtm2*). The experiment showed no enrichment of p*Lrrtm1* and p*Lrrtm2* in the immunoprecipitated DNA (Figure 11). This suggests that under these conditions, c-Jun is not binding to the regulatory regions of *Lrrtm1* and *Lrrtm2*, respectively. However, there was also no enrichment of p*Jun*, the promoter region of the *Jun* gene, which was previously shown to be autoregulated by its product, c-Jun (Angel et al., 1988). Consequently, until the provision of an adequate positive control for the binding of c-Jun,

no statement can be made in regard to its role in the transcriptional regulation of *Lrrtm1* and *Lrrtm2*.

Lrrtm1 and *Lrrtm2* expression and MeCP2

MeCP2 is an abundant protein in the nucleus of neurons (Skene et al., 2010), and is phosphorylated upon neuronal activity (Zhou et al., 2006; Buchthal et al., 2012). Zhou *et al*. hypothesised that this activity-induced phosphorylation leads to a de-repression of target genes (Zhou et al., 2006). Further, a microarray analysis of hypothalamic RNA from *MeCP2*-null and *MeCP2*-overexpressing mice, respectively, indicated that MeCP2 regulates a vast amount of genes; these included *Lrrtm1* and *Lrrtm2*, which were downregulated in the hypothalamus under MeCP2 overexpression conditions (Chahrour et al., 2008). In a developmental profile-experiment I could show that in cultured hippocampal neurons *Lrrtm1* and *Lrrtm2* mRNA levels continuously rise from the day of plating to day *in vitro* 21 (Figure 8A). Correlating with this rise is the amount of phosphorylated MeCP2 (Figure 8B). In order to further explore the role of MeCP2 and its activity-dependent phosphorylation in *Lrrtm1* and *Lrrtm2* expression, I infected hippocampal neurons with rAAVs expressing either wild-type MeCP2, $MeCP2^{WT}$, or $MeCP2^{S421A}$, a serine 421 non-phosphorylatable mutant form of MeCP2 (Figure 9). Both overxpression of wild-type and mutant MeCP2 lead to a decrease in basal expression of *Lrrtm2* (Figure 10A). However, the neuronal activity-induced upregulation of *Lrrtm2* mRNA levels was compromised neither by overexpression of $MeCP2^{WT}$ nor of $MeCP2^{S421A}$ (Figure 11A). *Lrrtm1* mRNA levels did not change in either condition (Figure 10B, 11A). Similarly, mRNA expression of *Grin2a*, a gene previously shown to be regulated by MeCP2 in the mouse hypothalamus (Chahrour et al., 2008), was also not changed (Figure 10C). To further explore the connection of *Lrrtm2* and MeCP2 and the presumptive role of MeCP2 as transcriptional repressor, I cloned a rAAV coding for a MeCP2-fusion protein, VP16-MeCP2. The VP16 (viral protein 16) transcriptional activator domain renders fused proteins constitutively active; i.e. in case of a transcription factor acting as repressor, this would constitutively activate its target genes (Zhang et al., 2011). However, infection of cultured hippocampal neurons with rAAV-*VP16-MeCP2* had no

significant effect on *Lrrtm2* expression (Figure 11B). Therefore, no statement can be made whether and by which mechanism MeCP2 influences *Lrrtm2* expression.

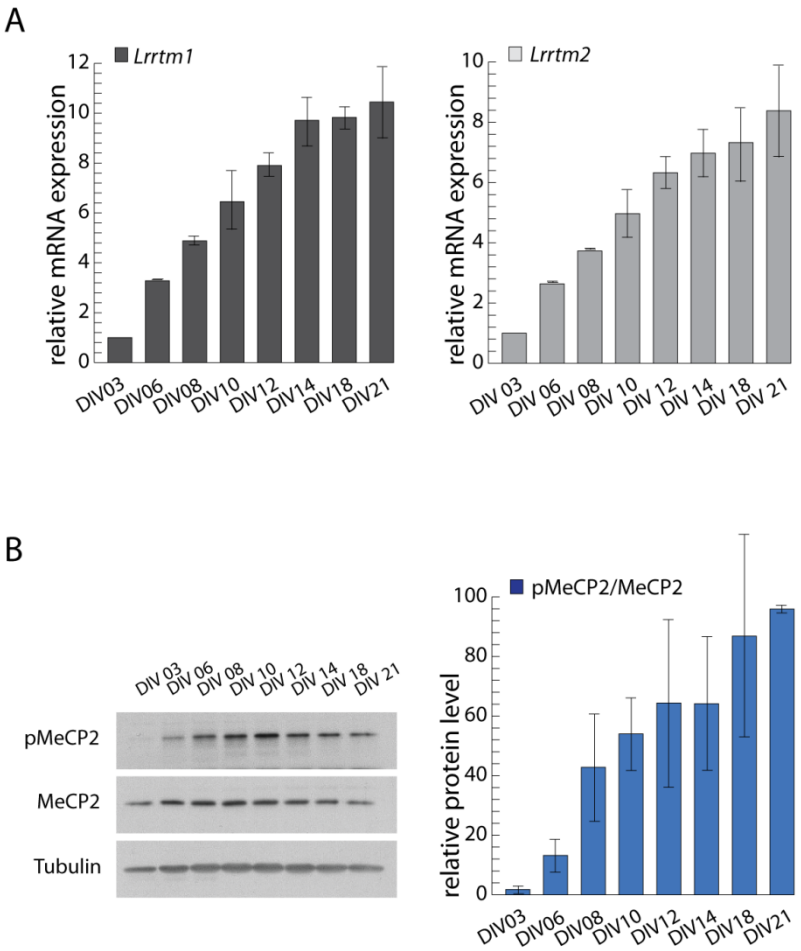

Figure 8. Developmentally regulated expression of *Lrrtm1* and *Lrrtm2* correlates with the amount of phosphorylated MeCP2.
A, Primary mouse hippocampal neurons were used to study the age-dependent basal expression of *Lrrtm1* and *Lrrtm2* over a period of three weeks. Endogenous *Lrrtm1* and *Lrrtm2* mRNA expression levels were measured on the indicated day *in vitro* by RT-qPCR using gene-specific primers. The expression level is expressed as fold increase of *Lrrtm1* and *Lrrtm2* mRNA levels over the levels measured on day *in vitro* 3. **B**, Neurons as in A were used for studying the age-dependent level of MeCP2 phosphorylation at serine 421 (pMeCP2) under basal conditions over the same period of time as in A. Neurons were harvested on the indicated day *in vitro* and the amount of total MeCP2 and pMeCP2 was determined by western blot. The *left panel* shows a representative western blot image of MeCP2 and pMeCP2 protein levels on the days *in vitro* indicated. The *right panel* demonstrates a quantification of the pMeCP2 signal normalized to the signal of total MeCP2; the signals are shown as fold increase of phosphorylated MeCP2 relative to the amount determined on day *in vitro* 3 of the first experiment. Data from three independent experiments are represented as mean values ± SEM. DIV, day *in vitro*.

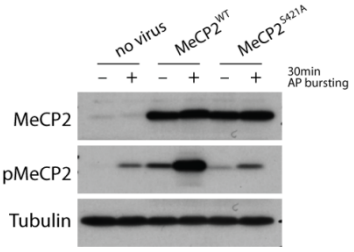

Figure 9. Expression of MeCP2 wild-type (WT) and mutant MeCP2 (S421A) in hippocampal neurons.
Primary mouse hippocampal neurons were infected on day *in vitro* 4 with rAAV-*MeCP2*^{WT} or rAAV-*MeCP2*^{S421A} leading to overexpression of the respective protein. Action potential bursting was induced by Bicuculline (50µM) on day *in vitro* 10 for the time indicated, followed by cell harvest and western blot analysis using antibodies to MeCP2 and phosphorylated MeCP2 at serine 421 (pMeCP2).

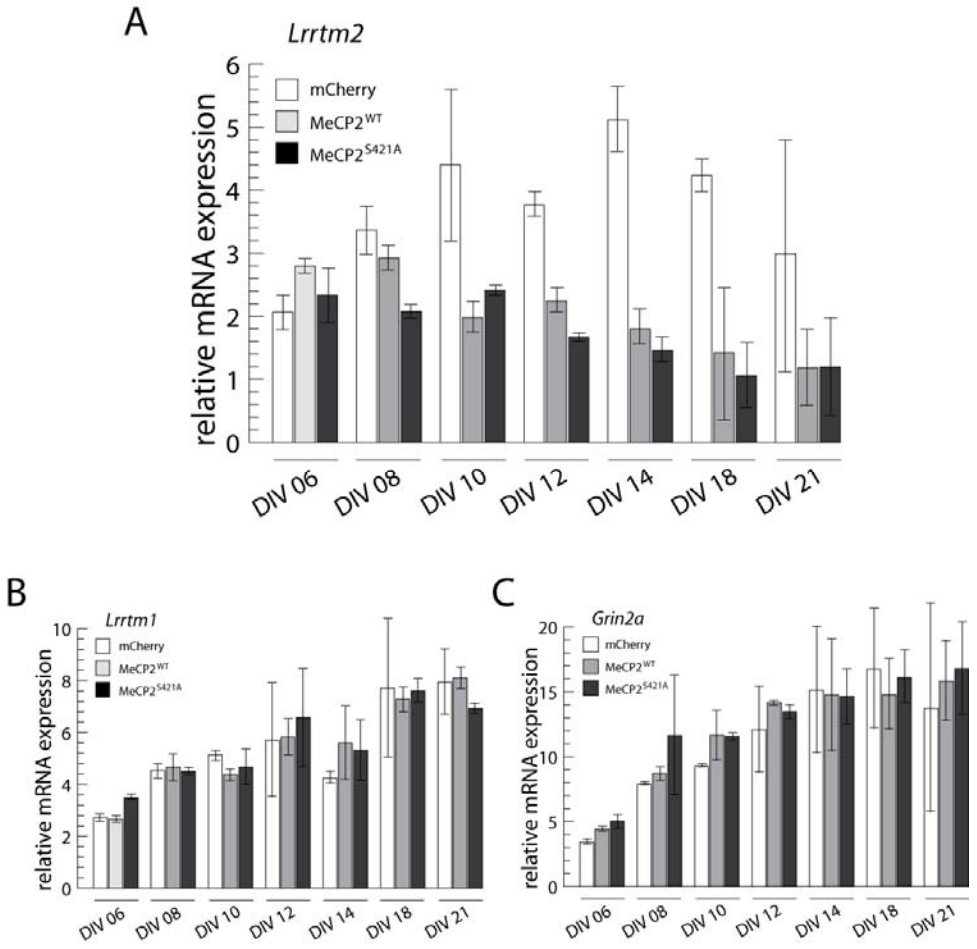

Figure 10. Overexpression of MeCP2 downregulates basal expression of *Lrrtm2*, but not of *Lrrtm1*.
A-**C**, Primary mouse hippocampal neurons were used for analysing the role of MeCP2 in the expression of *Lrrtm1* and *Lrrtm2*. **A**, Neurons were infected on day *in vitro* 3 with rAAV-*MeCP2*^{WT} and rAAV-MeCP2^{S421A}, respectively, or a control virus (*mCherry*) and the age-dependent basal expression of *Lrrtm2*, *Lrrtm1* and *Grin2a* was analysed over a period of three weeks. Endogenous *Lrrtm2* (**A**), *Lrrtm1* (**B**) and *Grin2a* (**C**) mRNA expression levels were measured on the indicated day in vitro by RT-qPCR using gene-specific primers. The expression level is expressed as fold increase of *Lrrtm2*, *Lrrtm1* and *Grin2a* mRNA level respectively, over the levels measured on day *in vitro* 3. Data represent mean values ± SEM from two independent experiments with duplicate measurements and are normalized to *Gusb*.

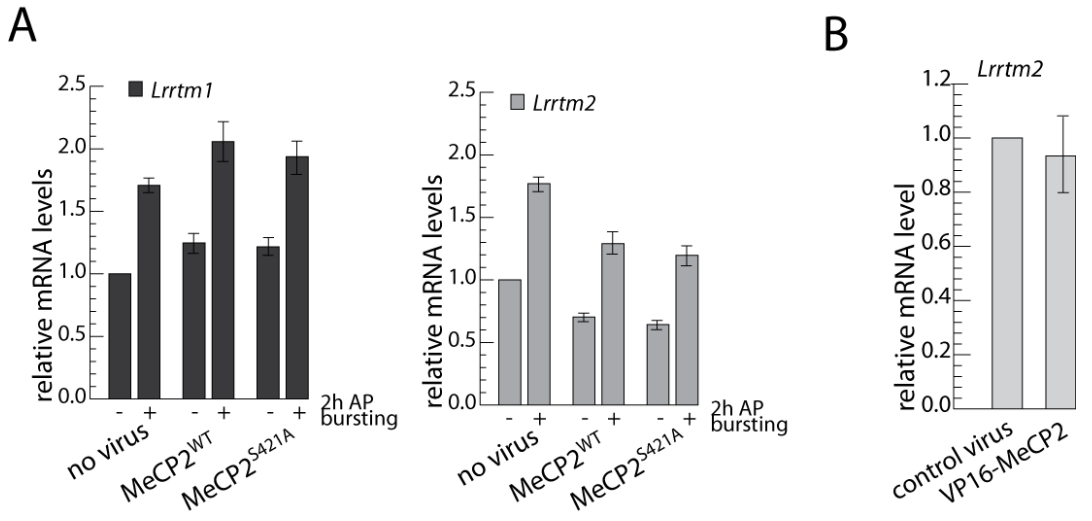

Figure 11. Overexpression of MeCP2 does not influence *Lrrtm1* and *Lrrtm2* mRNA induction upon AP bursting.

A, **B**, Primary mouse hippocampal neurons were used for analysing the role of MeCP2 in the expression of *Lrrtm1* and *Lrrtm2*. **A**, In neurons infected with rAAV-*MeCP2*^{WT} and rAAV-*MeCP2*^{S421A} respectively, action potential bursting was induced on day *in vitro* 10 using Bicuculline (50µM) for the time indicated. Expression levels of endogenous *Lrrtm1* and *Lrrtm2* mRNA were measured by RT-qPCR using gene-specific primers and are shown as fold induced levels of *Lrrtm1* and *Lrrtm2* mRNA over levels in untreated neurons. Data represent mean values ± SEM from six independent experiments with duplicate measurements and are normalized to *Gusb*. **B**, Neurons infected with rAAV-*VP16-MeCP2* or a control virus, rAAV-*VP16-EGFP* were harvested on day *in vitro* 10 and endogenous *Lrrtm2* mRNA levels were determined as described in A and are expressed as fold induced *Lrrtm2* mRNA level over the level under control conditions (control virus). Data represent mean values ± SEM from three independent experiments. DIV, day *in vitro*; AP, action potential.

Influence of *Lrrtm2* knock-down on spine density

The members of the Lrrtm protein family are capable of inducing presynaptic differentiation in neurons (Linhoff et al., 2009). Lrrtm2 shows the most potent synaptogenic capacity (Linhoff et al., 2009). As Bicuculline, via the activation of CREB, induces an increase in spine density (Papa and Segal, 1996) I hypothesised that the Bicuculline-induced, CREB-dependent upregulation of *Lrrtm2* might be responsible for the increase in spine density. This hypothesis was supported by the finding that overexpression of Lrrtm2 in mature neurons increases spine density (Linhoff et al., 2009), while shRNA (short hairpin RNA)-mediated knock-down of *Lrrtm2* mRNA was shown to lead to a decrease in spine density (de Wit et al., 2009). In order to interfere with the Bicuculline-

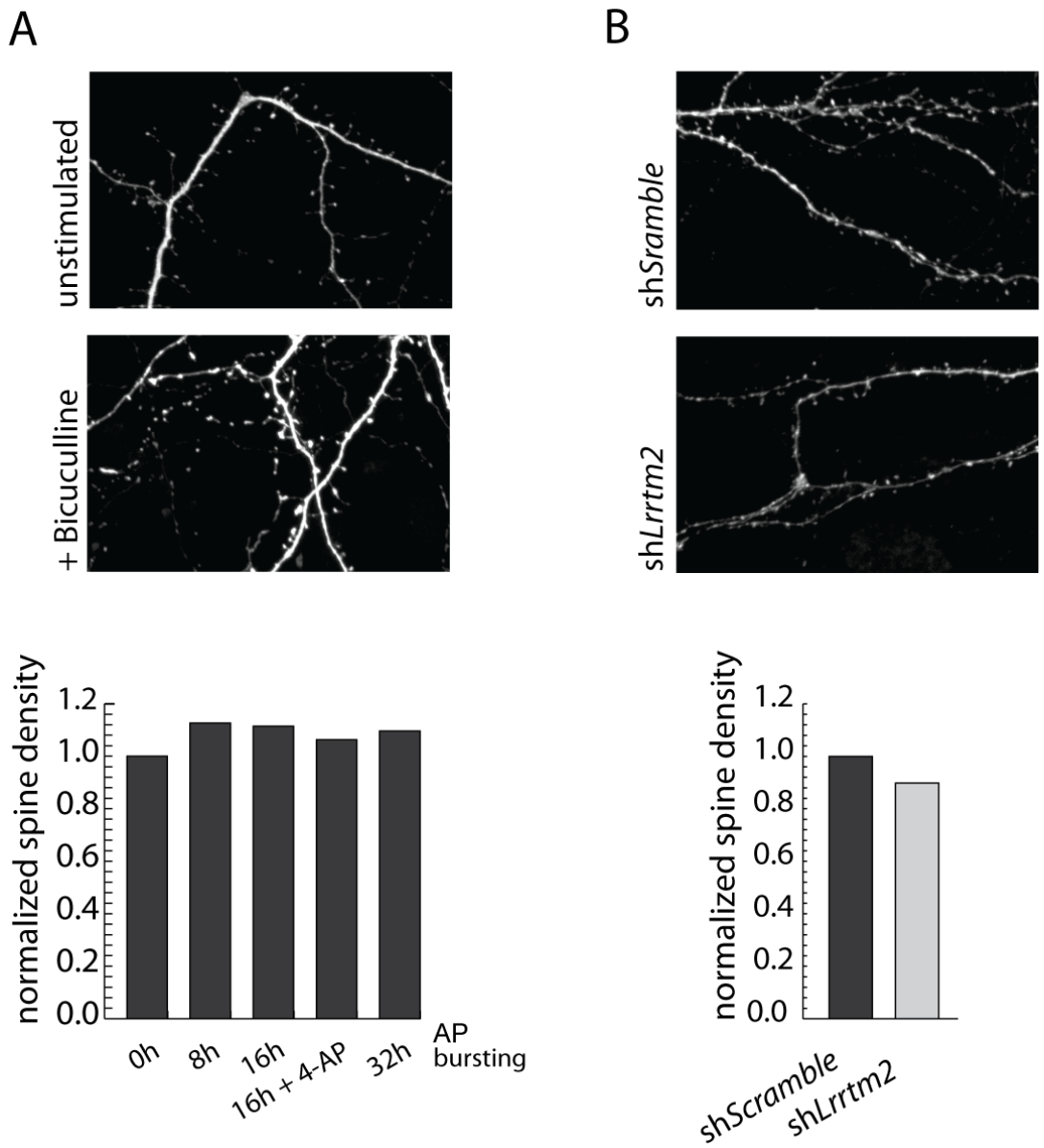

Figure 12. AP bursting and knock-down of *Lrrtm2* mRNA have no effect on spine density.
A, **B**, Primary mouse hippocampal neurons were used for determining spine density under different conditions. **A**, *upper panel*, Representative pictures of neurons that were either left untreated or subject to AP bursting using Bicuculline stimulation for 16h. **A**, *lower panel*, Quantification of spine density from neurons treated with Bicuculline to induce AP bursting for 0-32h. **B**, *upper panel*, Representative pictures of neurons that were infected with rAAV-sh*Lrrtm2* or a control virus, rAAV-sh*Scramble*. **B**, *lower panel*, Quantification of spine density from neurons infected with rAAV-sh*Lrrtm2* or a control virus, rAAV-sh*Scramble*. For visualization of individual cells, neurons were transfected with an expression vector for hrGFP. Data in lower panels are represented as spine density/20µm dendrite normalized to untreated neurons. AP, action potential.

induced upregulation of *Lrrtm2* mRNA, but not with basal levels, I aimed at creating shRNA sequences that resulted in a partial knock-down of the gene. I constructed three *Lrrtm2* knock-down vectors expressing different shRNA sequence targeting *Lrrtm2* mRNA. Among them was the *Lrrtm2* knock-down sequence previously published (de Wit et al., 2009) and a scrambled shRNA as control (Mauceri et al., 2011). Cultured mouse hippocampal neurons were infected with a rAAV carrying the *Lrrtm2* shRNA. For the visualisation of individual neurons, the cells were transfected with an expression vector coding for EGFP. In parallel, I analysed the spine density of hippocampal neurons before and after the induction of AP bursting using Bicuculline. Surprisingly, I could not detect a difference between the spine density of neurons that had been subject to AP bursting and untreated neurons (Figure 12A). Neither could see a significant difference in spine density between the neurons infected with the scrambled shRNA and the shRNA targeting *Lrrtm2* mRNA published by de Wit *et al*. (de Wit et al., 2009) (Figure 12B). At the same time, the group of Thomas Südhof presented data showing that *Lrrtm1*, *Lrrtm2*, *Neuroligin1* and *neuroligin3* are partially redundant and that a simultaneous knock-down of all four genes is necessary to reduce synapse numbers (Ko et al., 2011; Soler-Llavina et al., 2011). They attributed the previously observed effect on spine density by single knock-down of *Lrrtm2* (de Wit et al., 2009) to off-target effects of this specific shRNA sequence. Why these off-target effects did not occur under the conditions I applied remains elusive. The new data further suggested that Lrrtms fulfil a task in the function of the synapse rather than in their formation (Ko et al., 2011; Soler-Llavina et al., 2011; Soler-Llavina et al., 2013). Consequently, I did not further pursue experiments on spine density, but directed my focus to the role of *Lrrtm1* and *Lrrtm2* in neuronal network activity.

Influence of *Lrrtm1* and *Lrrtm2* knock-down on network activity

For the following experiment I collaborated with Dr. H. E. Freitag, a specialist in microelectrode array (MEA) recordings. To investigate whether *Lrrtm1* and *Lrrtm2* influence neuronal network activity we infected rat hippocampal cultures with rAAV expressing shRNA targeting *Lrrtm1* (rAAV-*shLrrtm1*), *Lrrtm2* (rAAV-*shLrrtm2*) or a control virus, rAAV-*shScramble* (Mauceri et al., 2011). We could observe that the number

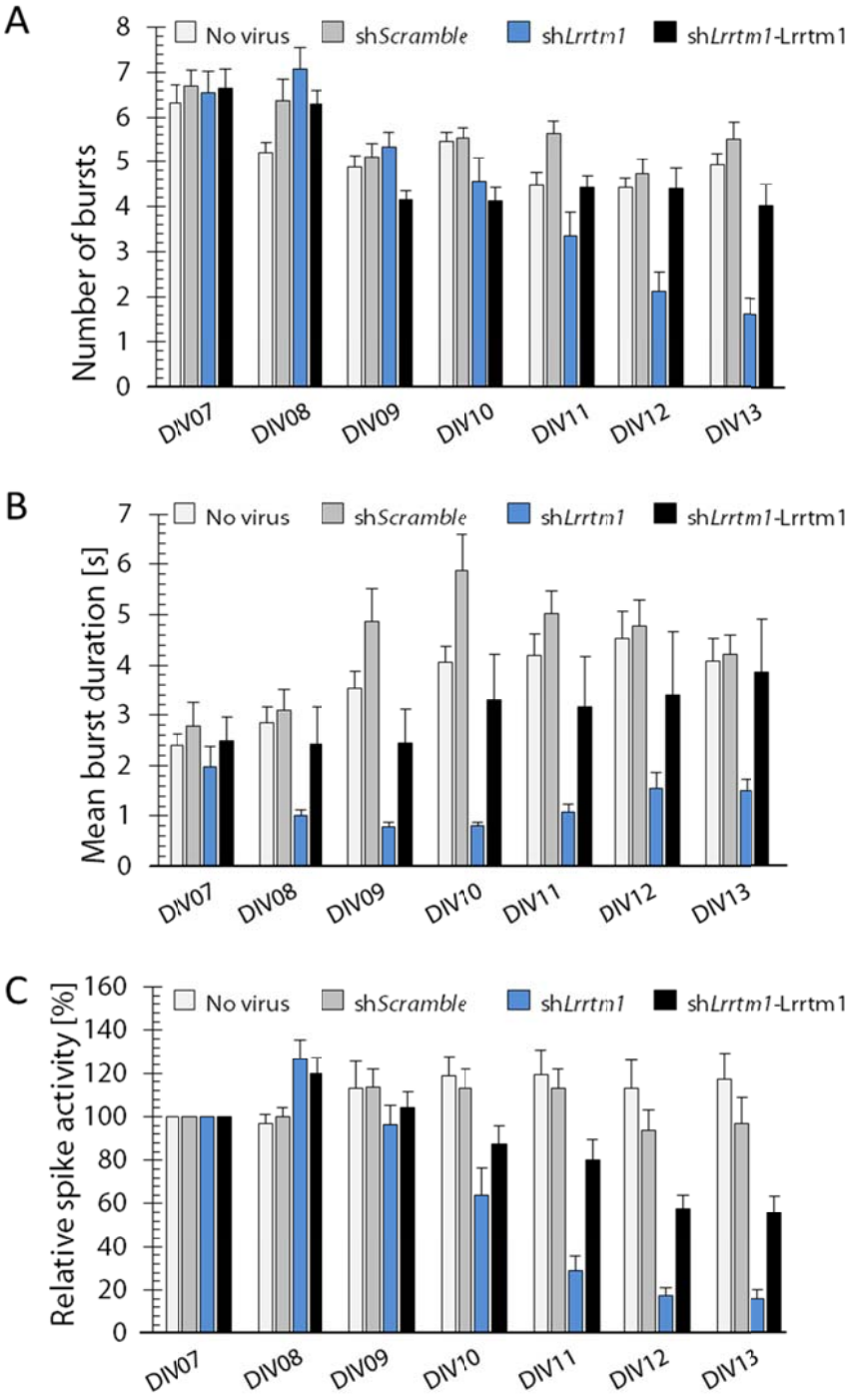

Figure 13. Knock-down of *Lrrtm1* mRNA reduces bursting activity.
A-**C,** Microelectrode array recordings of rat hippocampal cultures were used to study the impact of *Lrrtm1* mRNA knock-down on network activity. MEA analysis of number of bursts (**A**), mean burst duration (**B**), and relative spike activity (**C**) in uninfected cultures and cultures infected with rAAV-sh*Scramble*, rAAV-sh*Lrrtm1*, and the Lrrtm1 rescue virus rAAV-sh*Lrrtm1*-Lrrtm1. Neurons were infected on day *in vitro* 4. From day *in vitro* 7 to 13 recordings of spontaneous network activity were acquired for 5 min once a day. Bars represent mean + SEM of n=8-16 (A, B), n=8-11 (C) cultures. MEA, microelectrode array. DIV, day *in vitro*.

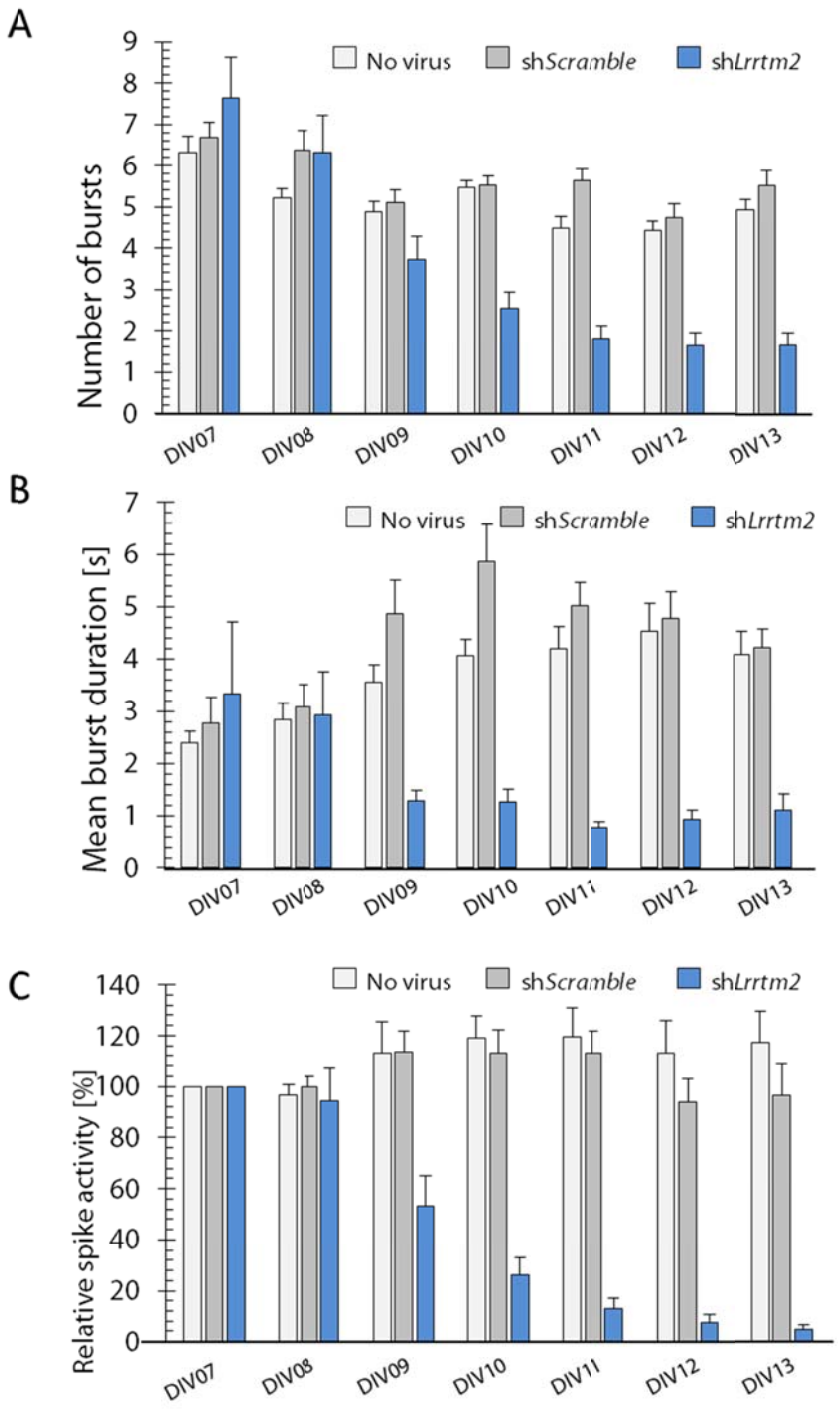

Figure 14. Knock-down of *Lrrtm2* mRNA reduces bursting activity.
A-**C,** Microarray recordings of rat hippocampal cultures were used to study the impact of *Lrrtm2* mRNA knock-down on network activity. MEA analysis of number of bursts (**A**), mean burst duration (**B**), and relative spike activity (**C**) in uninfected cultures and cultures infected with rAAV-sh*Scramble*, and rAAV-sh*Lrrtm2*. Neurons were infected on day *in vitro* 4. From day *in vitro* 7 to 13 recordings of spontaneous network activity were acquired for 5 min once a day. Bars represent mean + SEM of n=8-16 (A, B), n=8-11 (C) cultures. MEA, microelectrode array. DIV, day *in vitro*.

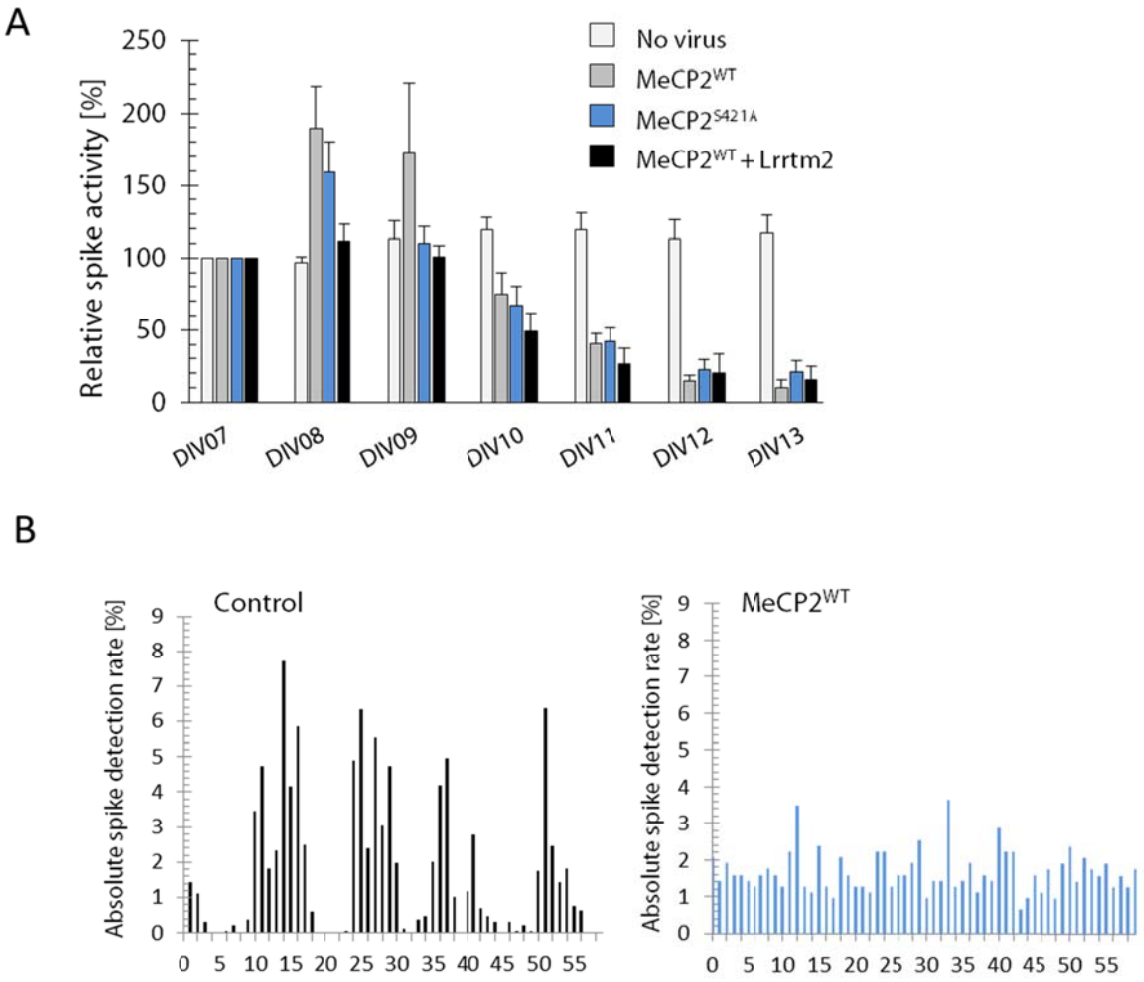

Figure 15. Overexpression of Lrrtm2 does not rescue MeCP2^{WT}-induced network changes.
A, **B**, Microarray recordings of rat hippocampal cultures were used to study the impact of MeCP2^{WT} and MeCP2^{S421A} overexpression on network activity. **A**, MEA analysis of relative spike activity in uninfected cultures and cultures infected with rAAV-*MeCP2*^{WT}, rAAV-*MeCP2*^{S421A}, or double-infected with rAAV-*MeCP2*^{WT} + rAAV-Lrrtm2. Neurons were infected on day *in vitro* 4. From day *in vitro* 7 to 13 recordings of spontaneous network activity were acquired for 5 min once a day. Bars represent mean + SEM of n=2-6 cultures. **B**, Representative recording of the absolute spike detection rate of control cultures and cultures infected with rAAV-MeCP2^{WT}. MEA, microelectrode array. DIV, day *in vitro*.

of bursts as well as the mean burst durations of cultures infected with rAAV-*shLrrtm1* was reduced compared to cultures infected with rAAV-*shScramble* (Figure X). The reduction caused by rAAV-*shLrrtm1* could be rescued by overexpression of Lrrtm1 protein (Figure 13). The decrease in network activity caused by infection with rAAV-*shLrrtm1* was first observed at day *in vitro* 10-11 (Figure 13), coinciding with the onset of robust *Lrrtm1* mRNA expression in vitro (see Figure 8A). Infection of hippocampal cultures with rAAV-*shLrrtm2* had similar effects on burst number and duration (Figure 14) and the reduction initially occurred at day in vitro 10, when *Lrrtm2* mRNA is strongly expressed (see Figure 8A).

Interestingly, neurons overexpressing wild-type MeCP2 ($MeCP2^{WT}$) or mutant MeCP2 ($MeCP2^{S421A}$) showed a similar change in the activity pattern, i.e. reduced spike activity. In a previous experiment I could show that overexpression of $MeCP2^{WT}$ lead to a decrease of *Lrrtm2* mRNA levels. Together these findings propose that reduction of *Lrrtm2* mRNA is responsible for the network changes in neurons that overexpress $MeCP2^{WT}$. To test this hypothesis we performed a double infection of hippocampal neurons with $MeCP2^{WT}$ and an expression vector for Lrrtm2. However, Lrrtm2 protein failed to rescue the $MeCP2^{WT}$-associated changes in network behaviour (Figure 15).

Lrrtm1 and *Lrrtm2* and AMPA trafficking

Arnold *et al.* have shown that stimulation of cultured hippocampal neurons with the $GABA_A$ receptor antagonist Bicuculline leads to synchronisation of the neuronal network activity (Arnold et al., 2005). It leads to an increase in bursting activity as well as an increase of the number of spikes within one burst (Arnold et al., 2005). The changes in network activity are persistent for at least 24h and depend on gene transcription, as treatment of the neurons with Actinomycin D, a potent inhibitor of gene transcription, abolishes the stable change in network activity (Arnold et al., 2005). Accordingly, in order to sustain the alteration in network behaviour after Bicuculline treatment, gene transcription is required. However, it remains elusive which gene(s) is necessary for the maintenance of the network changes. *Lrrtm1* and *Lrrtm2* are potential candidates for the following reasons;

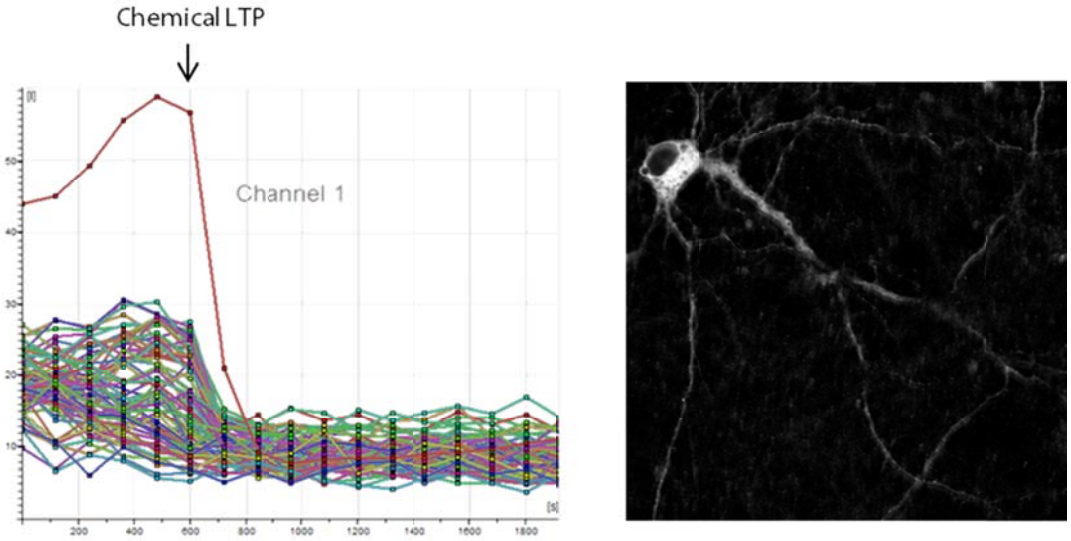

Figure 16. Chemical LTP does not lead to an increase in surface AMPAR fluorescence.
Mouse hippocampal neurons transfected with pCl-SEP-GluR2 (*right panel*) were live imaged under constant perfusion and fluorescence signals from 20 dendritic ROIs were quantified (*left panel*). Chemical LTP treatment was started at the indicated time point (*arrow*). ROI, region of interest.

first, as I show here they are regulated by Bicuculline-induced neuronal activity in hippocampal cultures. Second, the expression profile over time of *Lrrtm1* and *Lrrtm2* mRNA concurs with the time point where transcription is needed to sustain the changes in network activity (Arnold et al., 2005). Third, their respective proteins, Lrrtm1 and Lrrtm2, localize to the postsynaptic membrane of excitatory neurons (de Wit et al., 2009; Ko et al., 2009; Linhoff et al., 2009). Finally, Lrrtm1 and Lrrtm2 stabilize synaptic AMPA receptors and are required for LTP in mature synapses (Soler-Llavina et al., 2013). In order to find out whether the *Lrrtm1* and *Lrrtm2* induction is responsible for the stable synchronisation of the neuronal network, I transfected hippocampal neurons with pCl-SEP-GluR2. pCl-SEP-GluR2 is a plasmid coding for a fusion protein of the AMPA receptor subunit GluR2 and a pH-sensitive green fluorescent protein (pHluorin) (Kopec et al., 2006). This allows the visualization of AMPA receptor trafficking to the neuronal surface, as the pH value of the environment will change from acidic to neutral (Kopec et al., 2006). In the first step of the experiment, transfected neurons were stimulated by chemical LTP and live imaged

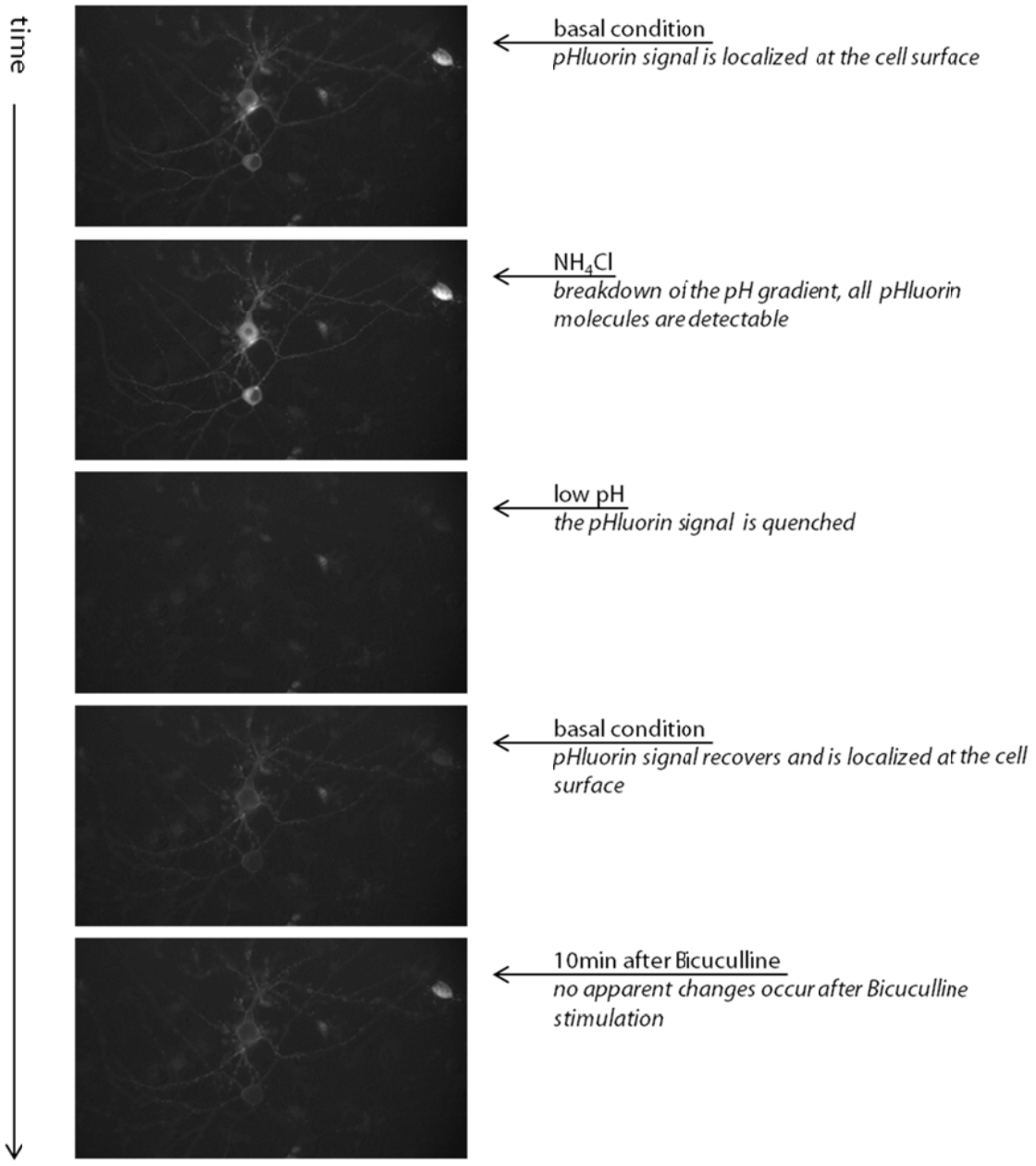

Figure 17. Bicuculline stimulation does not lead to an increase in surface AMPA receptor fluorescence.
Rat hippocampal neurons transfected with pCI-SEP-GluR2 were live imaged and fluorescence signals were detected over time under constant perfusion. NH_4Cl treatment and pH alteration served to test the SEP-GluR2 pH-dependency. A representative image sequence is shown.

using a confocal microscope detecting GFP, and a continuous flow of medium at room temperature. However, chemical LTP did not induce an increase in GFP fluorescence but contrariwise lead to a drop of the fluorescence signal (Figure 16). To exclude the possibility that room temperature is the preventive factor, I used a different microscopic setup with a continuous flow of medium at 37°C and Bicuculline stimulation to synchronise the network activity. Nonetheless I was still unable to detect an increase in the GFP fluorescence signal after stimulation (Figure 17). To rule out the possibility that the transfected pCl-SEP-GluR2 is not functional, I used ACSF (artificial cerebrospinal fluid) at a pH of 6 to quench the fluorescence signal, as well as NH_4Cl to collapse pH gradients resulting in a bright fluorescence signal. The pH-dependency of the pHluorin was clearly visible (Figure 17). In order to circumvent the pH-dependency of the transfected GluR2/GFP-pHluorin fusion protein I fixed the transfected cells and used antibodies to GFP to determine the ratio of surface to total AMPA receptors before and 15min after

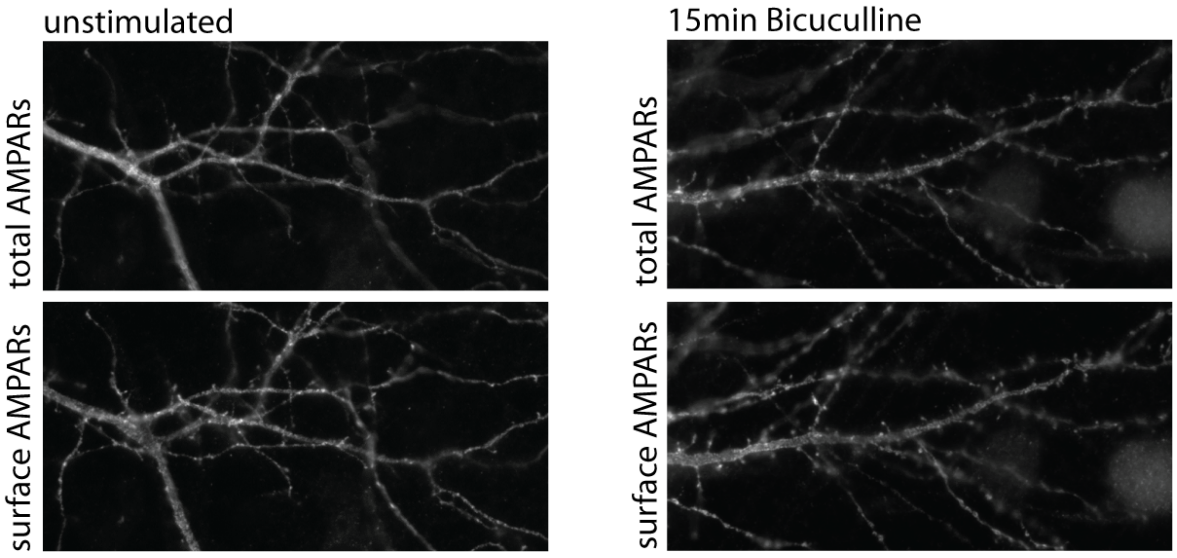

Figure 18. Staining of AMPA receptors before and after Bicuculline treatment.
Primary mouse hippocampal neurons were used for determining the amount of total- and surface-AMPA receptors under different conditions. Representative pictures of pCL-SEP-GluR2 transfected neurons that were either left untreated or subject to AP bursting using Bicuculline stimulation for 15min are shown. Neurons were stained before and after permeabilisation using an antibody to GFP and different fluorescent-labelled secondary antibodies to distinguish surface AMPAR fluorescence from total AMPAR fluorescence. AMPAR, AMPA receptor.

treatment with Bicuculline using two different methods, live cell staining and fixed cell staining. However, neither under these conditions was an increase in the relative surface AMPA receptor amount after stimulation with Bicuculline detectable (Figure 18). Consequently, either Bicuculline and chemical LTP stimulation of cultured hippocampal neurons do not induce trafficking of AMPA receptors to the neuronal surface under these conditions, or a more sensitive method to detect the fluorescence signal is required.

Discussion

The results of this study reveal several new findings about the recently identified synaptic cell adhesion molecule genes *Lrrtm1* and *Lrrtm2*. First, *Lrrtm1* and *Lrrtm2* expression is regulated by neuronal activity; they both are immediate-early genes whose activity-dependent increase in mRNA requires the activation of CaM kinases, CBP and nuclear calcium signals. Second, the induction of *Lrrtm2* expression depends on intact CRE sites immediately upstream of the gene, and its promoter region is bound by CREB. Third, knock-down of *Lrrtm1* and *Lrrtm2*, respectively, influences neuronal network activity. Finally, MeCP2 overexpression compromises basal mRNA expression of *Lrrtm2*, but not of *Lrrtm1*.

Physiological significance of activity-induced *Lrrtm1* and *Lrrtm2* expression

In this study, I analysed the neuronal activity-dependent regulation of *Lrrtm1* and *Lrrtm2* transcription in primary mouse hippocampal cultures and demonstrated that *Lrrtm1* and *Lrrtm2* mRNA levels increase about two fold upon Bicuculline-induced action potential bursting. My results show a slight difference in the time course of upregulation, with *Lrrtm2* mRNA levels having a peak induction after two hours, while *Lrrtm1* mRNA levels peak after four hours. To the best of my knowledge, this shows for the first time that neuronal activity regulates the gene expression of synaptic cell adhesion molecules.

To date, knowledge about the functions of *Lrrtm1* and *Lrrtm2* in neurons includes a role in AMPA-mediated excitatory synaptic transmission and subtle effects on behaviour and memory performance in knock-out animals (Soler-Llavina et al., 2011; Takashima et al., 2011; Voikar et al., 2013). In humans, polymorphisms of *LRRTM1* could be linked to schizophrenia (Francks et al., 2007; Ludwig et al., 2009), and microdeletions affecting *LRRTM2* have been found associated with mild cognitive impairment and developmental delay (Kleffmann et al., 2012). The studies on *Lrrtm1* and *Lrrtm2* function that have been described in the literature so far have not taken into account the possibility that expression

of *Lrrtm1* and *Lrrtm2* could be regulated by neuronal activity. The results of my study show that *Lrrtm1* and *Lrrtm2* expression is developmentally regulated in neuronal cell cultures and that their expression is induced by synaptic activity. It is possible, but not mandatory, that *Lrrtm1* and *Lrrtm2* have different functions under basal and active conditions in neurons. In the following, three possible functions of the latter, the *activity-induced* upregulation of *Lrrtm1* and *Lrrtm2,* are discussed.

(1) The concept of an analogy between neuronal and immune synapses was first established by M.A. Norcross (Norcross, 1984). It is based on structural as well as functional parallels, in that they both can be described as 'a stable adhesive junction between two cells across which information is relayed by directed secretion' (Dustin and Colman, 2002). The activation of T cells by antigen presenting cells requires an antigen-specific interaction between the T cell receptor and the MHC molecule on the surface of the antigen presenting cell (Janeway, 2001). It additionally requires a co-stimulatory signal mediated by molecules expressed on the membrane of T cells and antigen presenting cells (Janeway, 2001). The co-stimulatory signal increases the effectiveness of the T cell interaction in that it lowers the number of binding-triggered T cell receptors which is necessary for the activation (Viola and Lanzavecchia, 1996). A classic co-stimulatory interaction is CD28-CD80 (Bromley et al., 2001b; Bromley et al., 2001a), and there is also an inducible co-stimulatory molecule, ICOS (Hutloff et al., 1999). In drawing parallels to the neuronal synapse, a co-stimulatory signal may similarly exist to support synaptic transmission. Lrrtm1 and Lrrtm2 are possible candidates for a co-stimulatory signal, as they are inducible, and their induction depends on a preformed synaptic contact and synaptic activity. Since knockdown of *Lrrtm1* and *Lrrtm2* does not abrogate, but lower synaptic transmission (Soler-Llavina et al., 2011) one may reason that they enhance synaptic transmission, similar to the enhancement of T cell activation by co-stimulation.

(2) In a review on memory, Barco *et al.*, discuss a model in which memory formation involves several distinct molecular mechanisms whose respective location moves from the synapse to the nucleus and then back to the synapse (Barco et al., 2006). These include neurotransmitter release, activation of kinases and phosphatases, chromatin alterations, initiation of transcription factors and gene expression, synaptic capture of newly synthesised gene products and formation of new synapses (Barco et al., 2006). Involvement of activity-dependent expression of Lrrtm1 and Lrrtm2 in memory formation

would fit with such a model. Initiated by glutamate release and activation of NMDA receptors, the signal moves from the synapse to the nucleus via calcium signalling and CaM kinases, where it results in CREB-dependent gene transcription and possibly chromatin alterations mediated by CBP, resulting in upregulation of *Lrrtm1* and *Lrrtm2* mRNAs. From here the signal returns to the synapse by the translation of *Lrrtm1* and *Lrrtm2* mRNAs into proteins that are localized in the postsynaptic membrane, allowing the formation of new synapses, as has been shown by Linhoff *et al.* (Linhoff et al., 2009). Barco *et al*. further suggest that the molecular basis of memory persistence are self-perpetuating mechanisms that enable the neuron to maintain long-term changes which outlast the half-life of proteins (Barco et al., 2006). Examples of these mechanisms are prions, autophosphorylating kinases and AMPA receptor trafficking. They are based on the idea that a signal sustains itself by maintaining or reinforcing its source, similar to a perpetual motion machine or, more realistically speaking, a positive feedback mechanism. When applied to *Lrrtm1* and *Lrrtm2*, synaptic activity could be envisioned as source of the self-perpetuating system, and the Lrrtm genes and proteins as its tools. Thus, synaptic cell adhesion molecules could either be seen as a new example of a self-perpetuating machinery, or as part of the above mentioned mechanisms. Indeed, double knock-down of *Lrrtm1* and *Lrrtm2* during early postnatal life was found to selectively impair AMPA-mediated synaptic transmission (Soler-Llavina et al., 2011). This raises the possibility that the activity-induced expression of *Lrrtm1* and *Lrrtm2* partakes in inserting or stabilizing AMPA receptors in the postsynaptic membrane in response to LTP-inducing stimuli. This is supported by the finding, that Lrrtms are important for the maintenance of LTP (Soler-Llavina et al., 2013) Taken together, the characteristics of *Lrrtm1* and *Lrrtm2* make them very suitable for a role in memory, which will be interesting to explore.

(3) The groups of R.C. Malenka and T.C. Südhof both performed detailed experiments on the function of *Lrrtm1* and *Lrrtm2* using RNA interference-mediated loss of function approaches (Ko et al., 2011; Soler-Llavina et al., 2011). They could show that concurrent knock-down of *neuroligin1*, *neuroligin3*, *Lrrtm1* and *Lrrtm2* is required to reduce synapse numbers in hippocampal neurons *in vitro* (Ko et al., 2011). Most intriguing was the finding that this reduction depended on synaptic activity and CaM kinase signalling (Ko et al., 2011). Based on these results they proposed a model in which synapses are continuously eliminated and reformed in an activity-dependent manner as a proofreading mechanism,

and that the continuous elimination and reformation of synapses requires neuroligins and Lrrtms (Ko et al., 2011). The finding of this study that *Lrrtm1* and *Lrrtm2* are regulated by synaptic activity and CaM kinase signalling would fit with such a proofreading model. Further, it was recently shown that neuronal activity induces cleavage of neuroligin1, the postsynaptic ligand of neurexin (Peixoto et al., 2012; Suzuki et al., 2012). This mechanism might be an explanation for the finding that the elimination of synapses due to *neuroligin* and *Lrrtm* knock-down required activity (Ko et al., 2011). This is in line with the proofreading model postulated by Ko *et al*., and suggests that neuroligin is constantly produced and cleaved at active synapses, creating a perpetual turnover at the synapse. The balance of formation and elimination might be disrupted by excitotoxic conditions such as seizures, resulting in an excess cleavage of neuroligin1 which results in depression of synaptic transmission (Peixoto et al., 2012). The increased level of neuroligin1 cleavage and loss of synaptic transmission could either be a negative, toxic effect of overstimulation, or a cellular mechanism of protection against excitotoxicity. The activity-dependent cleavage of neuroligin1 was shown to require NMDA receptor activation and CaM kinase signalling (Peixoto et al., 2012; Suzuki et al., 2012), both of which are also involved in the activity-dependent regulation of *Lrrtm1* and *Lrrtm2*. Lrrtm1 and Lrrtm2 are further able to bind neurexin (de Wit et al., 2009; Ko et al., 2009; Siddiqui et al., 2010); this raises the possibility that Lrrtm1, Lrrtm2 and neuroligin1 act synergistically to maintain normal synaptic transmission. A model could be envisioned in which Lrrtm1 and Lrrtm2 serve as placeholders in the postsynaptic membrane for cleaved neuroligin1 to sustain neurexin clusters at the presynaptic side. The activity-dependent upregulation of Lrrtm1 and Lrrtm2 expression could serve as backsignal to the preceding neuron, conferring the information that a functional contact is formed and maintained, in line with the proofreading model.

Regulation of genetic modules through the signalling cascade of neuronal activity–nuclear calcium–CREB

The results of this study demonstrate that neuronal activity induces the expression of *Lrrtm1* and *Lrrtm2*. The upregulation of *Lrrtm1* and *Lrrtm2* mRNA requires intact function

of CaM kinases and nuclear calcium signalling. This pathway is one of the four major neuronal activity-controlled and gene-inducing signalling cascades, which comprise the MEK/ERK, p38, calcineurin and CaM kinase pathway. The latter, specifically CaMKIV signalling, requires nuclear calcium signals and has previously been shown to regulate the expression of distinct genetic programmes through the activation of the transcription factor CREB (Zhang et al., 2009; Zhang et al., 2011). CREB, in turn, was found implicated in many functions of the central nervous system, particularly in neuronal survival, memory formation, addiction, and neurogenesis (Carlezon et al., 1998; Impey et al., 1998; Pittenger et al., 2002; Zhu et al., 2004; Zhang et al., 2009; Zhang et al., 2011). In this study I found that the expression of *Lrrtm2* requires nuclear calcium signalling, and the promoter of the gene is bound and controlled by CREB. Consequently, one may speculate whether *Lrrtm2* contributes to one of the known roles of CREB in the central nervous system or is part of a new CREB-mediated function in neurons. The finding that mutations in the genes encoding *Lrrtms* have comparatively subtle effects in humans (Kleffmann et al., 2012; Rocca et al., 2012), or in knock-out animals (Takashima et al., 2011; Voikar et al., 2013), gives rise to the notion that Lrrtms fulfil a task in the fine-tuning of synapses (Francks et al., 2007). This might represent an as yet unexplored function of CREB in the brain, and requires an understanding of possible functions of Lrrtm1 and Lrrtm2 on the synaptic level, some of which I discussed in the preceding section.

The role of the co-activator CBP and the modifier MeCP2 in *Lrrtm1* and *Lrrtm2* transcription

In this study I show that the mRNA levels of *Lrrtm1* and *Lrrtm2* depend on intact CBP function. Expression of E1A, a viral protein that binds to and interferes with normal function of CBP (Arany et al., 1995; Bannister and Kouzarides, 1995), led to a decrease in basal expression of *Lrrm1* and *Lrrtm2* mRNA and compromised their activity-induced upregulation. CBP is a transcriptional co-activator that interacts with a variety of transcription factors (Shiama, 1997), including factors with putative binding sites in the *Lrrtm1* and *Lrrtm2* promoters. CBP has histone acetyltransferase activity (Kalkhoven, 2004) and is implicated in posttranslational modification of histones during learning

(Korzus et al., 2004). Also, like the expression of *Lrrtm1* and *Lrrtm2*, nuclear calcium and CaM kinases control CBP activity (Chawla et al., 1998). The finding that interference with CBP function influences both the basal and activity-induced expression of *Lrrtm1* and *Lrrtm2* raises the possibility that CBP regulates *Lrrtm1* and *Lrrtm2* expression either by cooperating with different transcription factors under basal and active conditions; or that the basal state of neurons, in which they are spontaneously active, is sufficient to stimulate CBP-dependent transcription. A transcription factor known to cooperate with CBP is CREB (Chrivia et al., 1993), whose binding to the promoter of *Lrrtm2* I could show in this study. CREB and CBP form a prototypical neuronal activity-controlled transcription regulating complex (Chawla et al., 1998; Hardingham et al., 1999; Hardingham et al., 2001b), which makes it likely that the disturbance of activity-induced *Lrrtm2* mRNA upregulation in the presence of the CBP inhibitor E1A is due to a failed interaction of CBP and CREB. The finding that in the presence of E1A the basal expression of *Lrrtm2* is reduced by 50% may suggest that CREB is also regulating transcription under basal conditions, in which neurons are never fully silent (see Figure 2E). This may also explain the developmentally regulated increase of *Lrrtm2* expression, which I could show in this study.

The finding that CBP plays a role in the regulation of *Lrrtm1* and *Lrrtm2* raises the possibility that they are implicated in the aetiology of Rubenstein-Taybi syndrome, a rare but severe disorder that is caused by mutation of the *CBP* gene (Rubinstein and Taybi, 1963). Some of the symptoms of this syndrome, for example the difficulties in learning, could be explained by synapse dysfunction due to the aberrant regulation of *Lrrtm1* and *Lrrtm2*.

A disease whose symptoms partly overlap with those of Rubinstein-Taybi syndrome is a disorder caused by mutations in the transcription factor gene *MECP2*, Rett syndrome (Rett, 1966; Amir et al., 1999). In this study I could show that overexpression of wild-type MeCP2 compromises the developmentally regulated increase of *Lrrtm2* mRNA, but not the one of *Lrrtm1*. The selective influence of MeCP2 on gene expression suggests a direct or indirect connection between MeCP2 and *Lrrtm2* executed by several possible mechanisms. First, an indirect link could be the impairment of basal activity; neurons overexpressing MeCP2 show a lower bursting frequency and the bursts occur less coordinated in the neuronal network (see Figure 15). Assuming that the development of the neuronal network

is paralleled by increasing spontaneous bursting activity, and that this regulates the increase in *Lrrtm2* mRNA, the impact of MeCP2 overexpression on activity could explain its effect on *Lrrtm2* expression. However, the fact that *Lrrtm1* is likewise controlled by neuronal activity but does not change its mRNA level upon the expression of MeCP2, suggests that additional factors play a role in the regulation of *Lrrtm1* or in that of *Lrrtm2*, or both. Second, the results of this study show that in hippocampal cultures the phosphorylation of MeCP2 continuously rises during the first two weeks after plating. Correlating with this rise is the increase in *Lrrtm1* and *Lrrtm2* mRNA expression. This suggests the possibility that phosphorylation of MeCP2 plays a part in the regulation of *Lrrtm1* and *Lrrtm2*. The finding that overexpression of MeCP2 impairs the increase of *Lrrtm2* mRNA could be based in a dominant negative function of MeCP2, sequestering the kinase activities responsible for the phosphorylation of MeCP2. However, this study also shows that overexpression of $MeCP2^{S421A}$, a mutant form of MeCP2 which cannot be phosphorylated at serine 421, still compromises the expression of *Lrrtm2*, suggesting that this phosphorylation site of MeCP2 has no function in the supposed regulation of *Lrrtm2*. Yet, since MeCP2 has several phosphorylation sites (Tao et al., 2009; Gonzales et al., 2012), this finding does not rule out a role of MeCP2 phosphorylation in the regulation of *Lrrtm2*. Finally, another possible direct link between MeCP2 and *Lrrtm2* might be CREB. MeCP2 was proposed to regulate transcription via interaction with CREB (Chahrour et al., 2008). As I have shown that CREB binds to the promoter of *Lrrtm2* (see Figure 6), the overexpression of MeCP2 might interfere with a normal function of the MeCP2-CREB interaction, resulting in an impaired expression of *Lrrtm2*. Taken together, the finding that high amounts of MeCP2 compromise the expression of *Lrrtm2* suggests that aberrant levels of Lrrtm2 contribute to the symptoms of Rett syndrome, which in some individuals is caused by a dublication mutation of *MECP2* (Das et al., 2013).

Experimental considerations

I would like to discuss several aspects of the experimental condition of this study.

First, an important question which was not addressed here is whether the observed upregulation of *Lrrtm1* and *Lrrtm2* mRNA translates to protein level. This could not be achieved due to the lack of good antibodies for Lrrtm1 and Lrrtm2.

Second, the Luciferase reporter assay required an enhancement of Bicuculline-induced action potential bursting in order to detect the Firefly luciferase signal. This raises the possibility that, in addition to CRE, there are other transcription factor binding sites in the promoter of *Lrrtm2* that contribute to the Bicuculline-induced upregulation of *Lrrtm2* mRNA *in vitro* which reside in a part of the promoter that was not included in the 356bp fragment cloned in the reporter vector. However the finding that Bicuculline stimulation in the presence of 4-AP lead to an induction of the firefly luciferase signal by 1.5 fold shows that the regulatory sites present in the cloned promoter fragment are *sufficient* for an induction of *Lrrtm2*. In addition the assay shows that the two CRE sites in the promoter fragment are *necessary* for the induction, as their mutation lead to an abrogation of the activity-induced signal.

Third, the signals detected in the qPCR analyses of immunoprecipitated DNA in the chromatin immunoprecipitation experiment correspond to gene-specific primers. Since there is a CRE site (CGTCA) in the untranslated region of exon1 of *Lrrtm1*, the primers that were used in the qPCR analyses to detect DNA bound by CREB are specific for this region. This means, however that they do not directly bind to the region which is commonly seen as promoter region, but a sequence approximately 350bp downstream. As the fragments of sheared chromatin constitute of around 500-1000bp, this still allows detection of the proximal promoter. However, in order to correctly compare the CREB binding to the promoters of *Lrrtm1* and *Lrrtm2*, primers specific for this region should be used. Another issue that was encountered in the chromatin immunoprecipitation experiment is that I was unable to find an adequate positive control gene for the binding of c-Jun. This is due to the fact that c-Jun is commonly seen as transcription factor induced by stress signals (Kyriakis et al., 1994). Consequently most of the target genes shown to be regulated by c-Jun so far are probably not targeted under the conditions that were applied in this experiment, namely synaptic activity. Due to the lack of the positive control gene I cannot rule out the possibility that the c-Jun antibody used in this experiment does not work and therefore the binding of c-Jun to *Lrrtm1* and *Lrrtm2* could not be detected, but actually is there.

Fourth, I was unable to find a positive control gene for the fusion construct VP16-MeCP2, which is supposed to constitutively activate MeCP2 target genes. I tested *Grin2a* and *Bdnf*, two genes previously shown to be regulated by MeCP2 (Zhou et al., 2006; Chahrour et al., 2008), but both did not show altered expression in VP16-MeCP2 overexpressing neurons (data not shown). This is either due to a non-functional VP16-MeCP2 construct or protein, respectively, or the genes are not targeted by MeCP2 under the conditions of the experiment. The latter is supported by the finding that *Grin2a* mRNA expression did also not change upon the overexpression of wild-type MeCP2, which raises the possibility that this gene is differentially regulated in the hippocampus and the hypothalamus, the region which was analyzed by Chahrour *et al.* (Chahrour et al., 2008).
Fifth, detection of the AMPA receptor trafficking proved to be very difficult. To persue this experiment, it would be necessary to set up reliable conditions to detect AMPA receptor trafficking, first by using an established stimulation protocol and then by Bicuculline stimulation. However, the setups available in our laboratory were not optimal for this purpose.

Future directions

The results of this study reveal a mechanism that links synaptic cell adhesion molecules to neuronal activity, both of which are important elements in synapse function. More work is needed to understand this connection and its contribution to normal brain function. It will be interesting to explore the physiological role of activity-induced expression of *Lrrtm1* and *Lrrtm2*, and to investigate their involvement in sustained adaptive changes in neurons and neuronal networks. This may help us to understand their part in the development of cognitive disorders and will add one little piece to the understanding of how our brain works.

Abbreviations

4-AP	4-aminopyridine
5q	chromosome 5, long arm
AAV	adeno-associated virus
ab	antibody
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor
APV	2-amino-5-phosphonovaleric acid
Atf3	activating transcription factor 3
BDNF	brain-derived neurotrophic factor
bp	base pair
Btg2	B cell translocation gene
CA	*cornu ammonis*
CaM	calcium/calmodulin
CaMBP	calcium/calmodulin binding peptide
CaMK	calcium/calmodulin-dependent kinase
cAMP	cyclic adenosine monophosphate
CASK	calmodulin-dependent serine protein kinase
CBA	chicken beta actin
CBP	CREB-binding protein
Ccrn4l	carbon catabolite repression 4-like
cDNA	copy DNA
c-fos	FBJ osteosarcoma oncogene
ChIP	chromatin immunoprecipitation
Jun	jun oncogene
CMV	cytomegalovirus
CR1	conserved region 1
CRE	cAMP/calcium response element
CREB	cAMP/calcium response element-binding protein

Dlg1	Drosophila disc large tumor suppressor
EF1α	elongation factor-1α
Eph	ephrin receptors
EPSC	evoked excitatory postsynaptic current
ERK	extracellular regulated MAP kinase
Fc	fragment, crystallizable
FLuc	firefly luciferase
GABA	γ-Aminobutyric acid
GADD45	growth arrest and DNA damage-inducible protein 45
GEFs	guanine nucleotide exchange factors
Gusb	glucuronidase, beta
HRP	horseradish peroxidase
ICOS	inducible T-cell co-stimulator
Ifi202b	interferon activated gene 202B
IgG	immunoglobulin G
IP3	inositol triphosphate
PAK	p21 activated kinase
LA	laminin
Lrrtm	leucine-rich repeat transmembrane
LTP	long-term potentiation
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MeCP2	methyl CpG binding protein 2
Mef2c	myocyte enhancer factor 2C
MHC	major histocompatibility complex
MINT	Munc 18 interacting protein
mRNA	messenger RNA
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NCAM	neural cell adhesion molecule
NFAT	nuclear factor of activated T-cells
NFκB	nuclear factor κB
NGLs	netrin-G-ligands

NMDA	*N*-methyl-D-aspartate
Npas4	neuronal PAS domain protein 4
Nr4a1	nuclear receptor subfamily 4, group A, member 1
P	postnatal
PCR	polymerase chain reaction
PDL	poly-D-lysin
PDZ	PSD95, post synaptic density protein, Dlg1, zo-1
PSD95	post-synaptic density protein 95
qPCR	quantitative PCR
rAAV	recombinant adeno-associated virus
RNAi	RNA interference
RT-qPCR	reverse transcriptase quantitative PCR
SALMs	synaptic adhesion-like molecules
SD	standard deviations
SEM	standard error of the mean
Serpinb2	serine peptidase inhibitor, clade B, member 2
SNP	single nucleotide polymorphism
SP1	specificity protein 1
SynCAM	synaptic cell adhesion molecule
Tiam	T cell lymphoma invasion and metastasis
TrkB	Tyrosine-related kinase B
USF	upstream stimulatory factor
VGLUT1	vesicular glutamate transporter 1
VP16	viral protein 16
WPRE	woodchuck posttranscriptional regulatory element
WT	wild-type
zo-1	zonula occludens-1 proteinCMV

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