

Isoform-specific regulation of behaviour by Caskin neuronal scaffold proteins

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List of abbreviations

AMPA: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ASD: autism spectrum disorder

CASK: calcium/calmodulin-dependent serine protein kinase

dHZ: double heterozygote

dKO: double knockout

DLG: Discs large homolog

GluA1: AMPA type glutamate receptor subunit A1

KO: knockout

MAGI: Membrane-associated guanylate kinase, WW and PDZ domain-containing protein

MAGUK: Membrane-associated guanylate kinase

mPFC: medial prefrontal cortex

MPP: palmitoylated membrane proteins

NMDA: *N*-methyl-D-aspartic acid

PMS: Phelan–McDermid syndrome

PSD: postsynaptic density

SCZ: schizophrenia

Shank: SH3 and multiple ankyrin repeat domains

TARP2: transmembrane AMPA regulatory protein

Scientific background

Activity dependent changes of synapses

A fundamental goal of neuroscience is to understand how the brain generates learned or adaptive behaviours that enable animals to thrive in complex environments. The central concept is that learning involves enduring, experience-driven changes in the brain that enhance the ability to perform critical tasks, such as acquiring essentials like food and shelter while avoiding harm from injury or predation. This behaviour is inherently complex, requiring accurate perception of inputs coming from the environment, the capacity to make appropriate decisions, and the ability to navigate effectively within the environment (Magee and Grienberger, 2020).

Synapses in the central nervous system undergo bidirectional modifications in synaptic strength, a process known as synaptic plasticity. These changes can occur locally in a homo- or heterosynaptic manner, as seen in long-term potentiation (LTP) or long-term depression (LTD) - collectively termed Hebbian plasticity - or globally during homeostatic scaling. Hebbian plasticity is widely regarded as the cellular foundation of learning and memory, while homeostatic scaling plays a critical role in the bidirectional regulation of neuronal excitability and in maintaining synaptic strength within a dynamic range (Diering and Huganir, 2018).

Glutamatergic excitatory transmission between synapses relies on receptors capable of binding glutamate. Two major classes of glutamate receptors have been identified in synapses, α -amino-3-hydroxy-5-methylisoxazole-4-propionic (AMPA) receptors and *N*-methyl-D-aspartic acid (NMDA) receptors, distinguished by their specific agonists. These receptors serve distinct roles in synaptic transmission. AMPA receptors are tetrameric ionotropic receptors composed of preassembled dimers formed from four closely related subunits, GluA1–4. In the adult mammalian central nervous system, the most prevalent dimers are GluA1/2 and GluA2/3. AMPA receptors are activated by the binding of the neurotransmitter glutamate, which opens their central channel. This allows the influx of sodium ions (and calcium ions, in the absence of the GluA2 subunit) and the efflux of potassium ions, resulting in depolarization of the postsynaptic neuron (Moretto and Passafaro, 2018).

Basal transmission is mediated by AMPA receptors, whereas NMDA receptors do not contribute to this process due to the voltage-dependent magnesium block of the channel pore. During elevated stimulation, strong depolarisation leads to the activation of NMDA receptors, allowing a transient influx of calcium into the postsynaptic compartment. Postsynaptic calcium is both necessary and sufficient for the induction of long-term potentiation (LTP), driving the

processes that enhance AMPA receptor mediated transmission (**Figure 1**) (Hayashi, 2021). Under basal conditions, AMPA receptors are continuously removed from the plasma membrane to recycling endosomes, and returned from them to the surface in a balanced way. During LTP, activation of NMDA receptors raises intracellular calcium levels, leading to increased AMPA exocytosis, and induces downstream processes leading to an increase in synaptic strength (Citri and Malenka, 2007). Moreover, Rab11a-dependent trafficking mediates the synaptic delivery of AMPA receptors triggered by LTP (**Figure 1**) (Citri and Malenka, 2007).

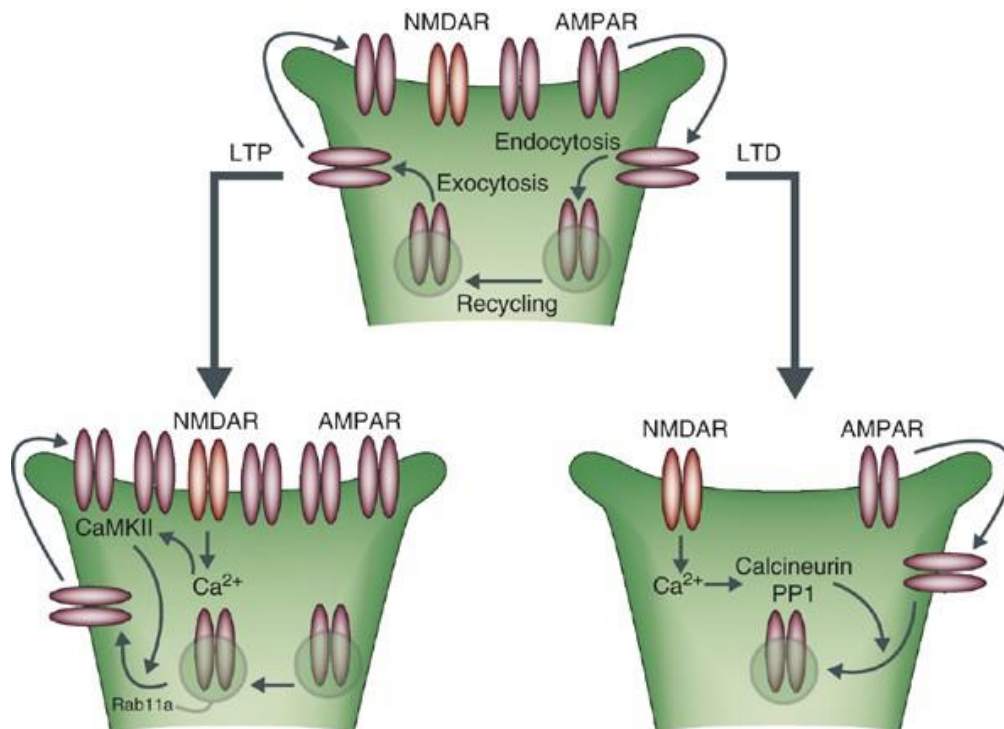


Figure 1. Major changes in ionotropic glutamate receptor distribution during LTP and LTD. During LTP, exocytosis of AMPA receptors (AMPA) is promoted by adaptor proteins and kinases. During LTD, the balance is shifted towards endocytosis by phosphatases and other downstream effectors (Citri and Malenka, 2007).

Similarly to LTP, long-term depression (LTD) is input-specific and depends on an NMDA receptor dependent increase in postsynaptic calcium levels. The prevailing hypothesis suggests that the quantitative properties of the calcium signal within dendritic spines determine whether LTP or LTD is triggered. Specifically, a modest increase in calcium triggers LTD, while a more substantial rise above a critical threshold induces LTP. Additionally, calcineurin (protein phosphatases) triggers LTD by stimulating AMPA receptor endocytosis (**Figure 1**). The discovery of silent synapses and their conversion to functional synapses during LTP, driven by the plasma membrane delivery of AMPA receptors, supported the idea that LTD involves the reverse process -namely, the endocytosis of AMPA receptors. Consistent with this

hypothesis, it was shown that pharmacological manipulations of neuronal activity or the application of glutamate receptor agonists can modulate this process in cultured neurons (**Figure 1**) (Citri and Malenka, 2007).

Long-term potentiation (LTP) relies on the enhancement of synaptic AMPA receptors. The threshold, induction mechanisms, magnitude, and stability of LTP are influenced by the specific types of AMPA receptors available for synaptic recruitment. According to the AMPA receptor code, a hierarchy exists for the selective incorporation of AMPA subunit and post-translational modification (PTM) combinations. This suggests that different forms of LTP preferentially recruit specific types of AMPA receptors (Diering and Huganir, 2018).

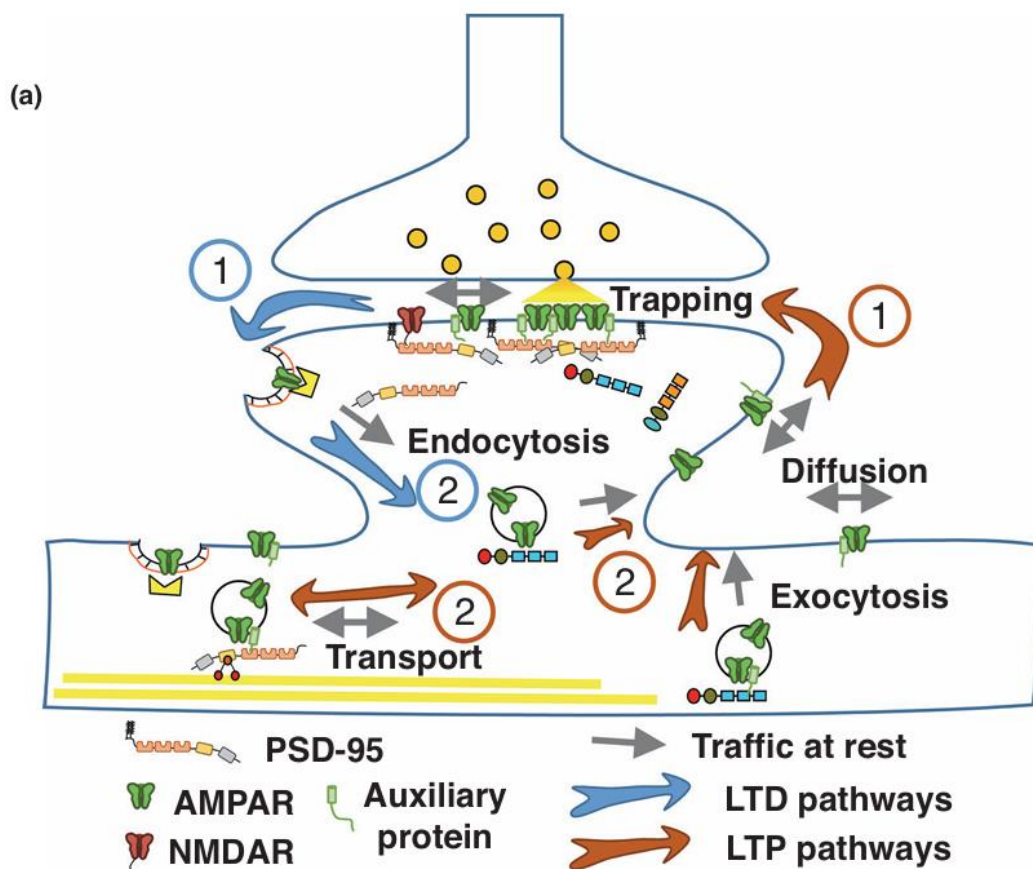


Figure 2. Possible pathways of AMPA receptor trafficking to and from the plasma membrane. Receptors enter the membrane via exocytosis, then move into the synaptic zone via lateral diffusion, with the help of scaffold proteins. Scaffold proteins also trap them in nanodomains. During both basal activity and LTD, they can be endocytosed from the membrane and either recycled later or degraded in lysosomes (Choquet and Hosy, 2020)

The PSD is an electron-dense structure located beneath the postsynaptic membrane of excitatory synapses, typically found within dendritic spines. The PSD in the mammalian brain

consists of a densely packed protein complex, forming a disk-like structure with a diameter of 200–800 nm and a thickness of 30–60 nm. Proteomic studies have identified many proteins within the PSD, including neurotransmitter receptors, cell adhesion molecules, scaffold proteins, signalling enzymes, cytoskeletal proteins, and membrane trafficking proteins (Kaizuka and Takumi, 2017).

The increase in AMPA receptor content within the PSD is initially supplied by non-synaptic pools of AMPA receptors diffusing laterally within the plasma membrane. These pools are subsequently replenished by the exocytosis of AMPA receptors from intracellular recycling endosomes. Processes such as exocytosis, lateral diffusion, PSD trapping (**Figure 2**), and alterations in single-channel properties are tightly regulated by multiple factors, such as post-translational modifications (PTMs), protein interactions and reversible phosphorylation. Localisation of receptors is controlled by various post-translational modifications that alter the structure of AMPA receptors and influence their binding to scaffold proteins known to participate in intracellular transport (Iasevoli et al., 2013). For example, phosphorylation of GluA1 at sites S831 and S845 is strongly associated with long-term potentiation (LTP). NMDAR-dependent LTP involves calcium influx, which activates CaMKII (calcium/calmodulin-dependent protein kinase II) and PKC (protein kinase C). These kinases directly phosphorylate GluA1 at S831, enhancing GluA1 single-channel conductance and promoting its recruitment to the PSD (Diering and Huganir, 2018).

The intracellular C terminal tail of AMPA receptors primarily facilitates plasma membrane directed trafficking but does not appear to play a role in lateral diffusion or the anchoring and positioning of these receptors. Instead, it is more likely that AMPA receptor auxiliary proteins, which interact with postsynaptic density (PSD) scaffold proteins, mediate activity-dependent changes in AMPA receptor positioning (**Figure 2**) (Droogers and MacGillavry, 2023).

Synaptic scaffold proteins

A specialized class of organizer proteins known as scaffold proteins efficiently construct multi-component signalling networks within the synapses. These proteins: *(i)* recruit multiple proteins to specific locations, *(ii)* arrange them into higher-order macromolecular complexes, *(iii)* facilitate their interactions while fine-tuning activity and crosstalk among the assembled proteins, and *(iv)* coordinate the functions of distinct molecular assemblies within various cellular regions or "microdomains". Scaffold proteins leverage their diverse domain

architecture, specific compartmentalization, and intrinsic protein dynamics to not only facilitate interactions among multiple partner proteins but, in some cases, also alter the properties of the assembled complexes (Pan et al., 2012). Acting as dynamic platforms for protein assembly, postsynaptic scaffolds enable crosstalk between different signalling pathways and integrate a variety of biochemical stimuli. With their distinct architecture of modular protein domains, precise subcellular localization, and ability to undergo protein modifications, scaffold proteins are recognized as active signalling hubs rather than passive structural platforms (Pan et al., 2012).

The MAGUK protein family

Membrane-associated guanylate kinases (MAGUKs) can be categorized into several subfamilies. The DLG subfamily comprises of SAP97 (DLG1), PSD93 (DLG2), SAP102 (DLG3) and PSD95, which are pivotal in regulating glutamatergic synaptic signalling and apical–basal cell polarity. CASK, belonging to another subfamily, is found on both the pre- and postsynaptic sides of excitatory synapses, influences synaptic vesicle trafficking, neurotransmitter release, and postsynaptic signalling. The MAGI subfamily proteins are expressed across various tissues, including the brain (e.g., MAGI2 at inhibitory synapses) and epithelial tissues, where they play essential roles in tissue development and function. The MPP subfamily is integral to establishing and maintaining cell polarity in diverse cell types, including neurons (Zhu et al., 2016).

MAGUKs are among the most extensively studied scaffolding proteins. Recent findings highlight the diverse roles of PSD-95 and other MAGUKs in regulating synaptic receptor expression, mediating synaptic plasticity, and maintaining the fundamental structure of the PSD. Light and electron microscopy studies suggest that a significant percentage of synapses contain both PSD-93 and PSD-95. Further investigation using super-resolution microscopy has revealed subsynaptic nanodomains at many synapses that are enriched with PSD-95. These nanodomains are also highly enriched in AMPA receptors, suggesting a functional relationship between PSD-95 and AMPA receptor clustering. While studies have demonstrated that PSD-93 and PSD-95 perform similar roles in AMPA receptor trafficking, PSD-93-enriched nanodomains have not been observed, underscoring a unique spatial organization for PSD-95 (Won et al., 2017).

It is also important to note that during the development of synaptic structures, the interplay of postsynaptic scaffolds and AMPA receptors is pivotal. The role of PSD-95 as a scaffold protein for AMPA receptors has been a significant focus of research. Mechanistically,

PSD-95 stabilizes AMPA receptors at the synaptic surface by organizing nanodomains at the synapse that are preferentially enriched in AMPA receptors rather than NMDA receptors. This clustering within nanodomains, which enhances synaptic efficacy and is activity-regulated, depends on the formation of a complex with the transmembrane AMPA regulatory protein (TARP) $\gamma 2$ (stargazin). This complex is crucial for the proper targeting and diffusion of AMPA receptors to the synaptic surface. Additionally, PSD95/stargazin complexes are thought to regulate AMPA receptor "slots" at the synapse, where freely diffusing AMPARs are "trapped" to modulate synaptic strength (Bissen et al., 2019).

SHANK proteins, the master scaffolds of the postsynaptic site

SHANK proteins serve as "master" scaffolding proteins that anchor and organize intermediate scaffolding proteins. They are found at excitatory synapses, where they play a critical role in synaptic development and function. In mice, SHANK proteins are encoded by the *Shank1*, *Shank2*, and *Shank3* genes. The expression patterns of the three SHANK protein family members differ slightly in the rodent brain. For instance, SHANK3 is highly enriched at corticostriatal glutamatergic synapses. *Shank1* mRNA appears to be exclusively expressed in the brain and is particularly abundant in regions such as the cortex, thalamus, amygdala,

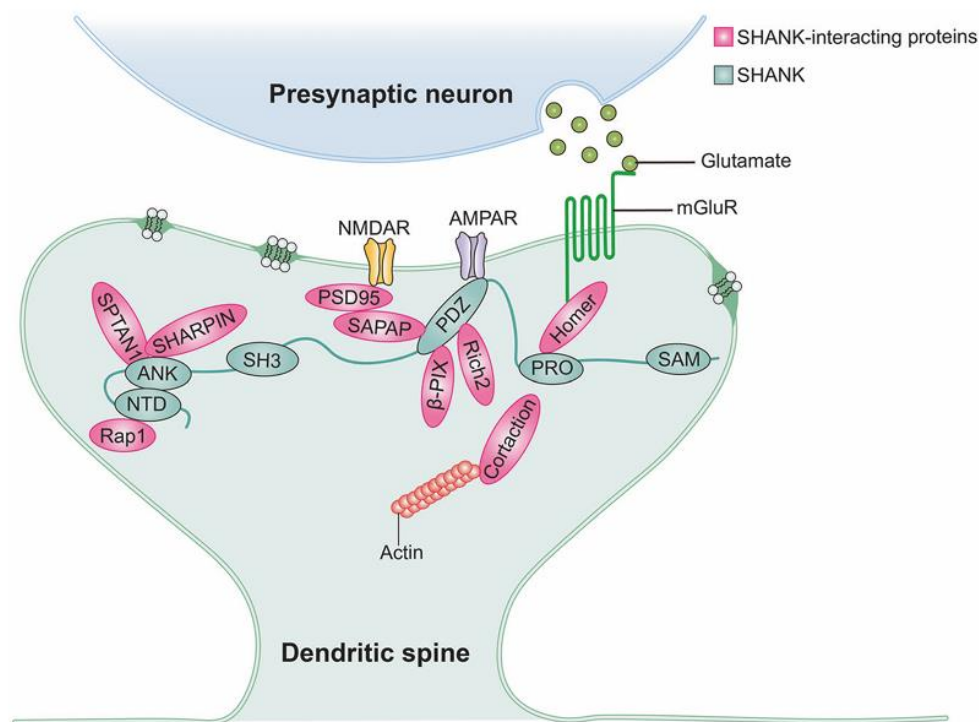


Figure 3. The domain structure of Shank with its binding partners at the postsynaptic site. Shank through its domains is capable of binding a large variety of proteins, making it an important postsynaptic scaffold (Liu et al., 2022).

hippocampus (CA1 and CA3 and the dentate gyrus), and cerebellar Purkinje cells. *Shank2* mRNA is enriched in similar brain regions, but also appear in other organs, such as the kidneys (Monteiro and Feng, 2017).

Within the postsynaptic area, glutamate receptors bind directly or indirectly to Shank proteins. NMDA-type glutamate receptors connect to the Shank PDZ domain through multiple associated proteins, which link the PSD-95-NMDA receptor complex. AMPA-type glutamate receptors associate with the Shank complex via PSD-95's interaction with stargazin. Metabotropic glutamate receptors (mGluRs) and inositol triphosphate receptors (IP3Rs) link to the Shank protein complex via Homer proteins, which bind to Shank's proline-rich region (**Figure 3**) (Sala et al., 2015).

Caskin isoforms: similarities and differences

Caskin proteins were discovered through their ability to interact with calcium/calmodulin-dependent serine protein kinase (CASK), a multidomain adaptor protein. Caskin proteins are present in two isoforms, Caskin1 and 2, which show high amount of homology, and are seemingly evolutionary conserved, but are located on chromosome 17 and 11, respectively. The N-terminal part of Caskins features a multidomain architecture that includes six ankyrin repeats, a single SH3 domain, and two tandem SAM domains while the C-terminal half contains a long proline-rich sequence and a distinct, conserved C-terminal domain (CTD). The N-terminal regions of Caskin1 and Caskin2 are highly similar, particularly within their identified domains. In contrast, the C-terminal regions differ significantly but include conserved patches of sequence, often centred around proline residues (Tabuchi et al., 2002). After the initial description, a further difference was identified, as only Caskin1 possesses a Cask binding domain between the SH3 and tandem SAM domains (Stafford et al., 2011).

Caskin1 isoform is expressed predominantly in the brain, with similar levels within specific regions, while weak expression is detected in the peripheral nervous system or in the testis. Caskin1 was reported to be slightly enriched within the postsynaptic density fraction (Katano et al., 2018). As for Caskin2, it is expressed widely in different adult tissues but without any specific enrichment within the CNS ((Human Protein Atlas; <https://www.proteinatlas.org/ENSG00000177303-CASKIN2/tissue>). Currently, most research is focused on Caskin1 isoform, with less amount of attention going towards Caskin2 but neither functional redundancy nor isoform-specific features have been reported so far between the isoforms.

Regarding Caskin1, it has been shown that the Nck family of adaptor proteins recruits them to be phosphorylated. Nck possesses three SH3 domains, which can only bind to Caskin1 when all are present based on immunoprecipitation experiments. Nck then recruits receptor tyrosine kinase EphB1 through Nck's SH2 domain. The role of EphB receptors in spine development is well established, with earlier studies demonstrating their presence in the postsynaptic structures of dendrites (Pesti et al., 2012).

Interestingly, further research revealed that the SH3 domain is unable to bind proline rich ligands, but instead it binds lysophosphatidic acid (LPA), which is a lipid frequently used as a binding site for proteins, affecting a wide range of functions, as well as inducing membrane curvature. The canonical SH3 domain contains multiple highly conserved aromatic residues, present in almost all cases. However, in case of Caskin1 key residues in the protein binding pocket are switched from aromatic residues to small lipophilic residues. Similarly to Caskin1, the NMR structure of the Caskin2 SH3 domain also reveals a unique ligand-binding cleft populated with charged amino acids, and the same lack of aromatic residues. Based on tryptophan fluorescent measurements the SH3 domain of Caskin1 selectively binds oleoyl LPA. In the case of Caskin2 the distinct features in its SH3 domain led to the hypothesis that the bind to a non-canonical ligand, rather than the typical proline-rich sequences usually recognized by this domain. Phage display experiments failed to identify a ligand for the Caskin2 SH3 domain, suggesting that it may indeed be non-functional (Kwan and Donaldson, 2016, Koprivanacz et al., 2017).

Interestingly, the SH3 domain of Caskin1 seems to completely lack the proline-rich binding groove that is required for canonical protein interactions. Selective binding to LPA enables the anchoring of the Caskin1 SH3 domain to membrane microdomains where LPA accumulates during specific signalling events. By acting as docking sites for the SH3 domain, lysophosphatidate within the plasma membrane may help direct Caskin1 interaction networks. For example, given the competing binding of Mint1- and Caskin1-CASK complexes to the cytoplasmic tail of neuexin, it can be hypothesized that a concurrent LPA signalling event might modulate the activity of this protein network (Tőke et al., 2021).

The SAM domain is a rather common interaction domain, which can facilitate interactions between proteins, RNAs and lipids. They commonly form hetero- or homodimers or polymers. Caskin1 was found to be able to polymerize in this manner, forming long helical fibres. At presynaptic sites, the size of the Caskin1 SAM polymer may be constrained by interactions with other proteins, as well as steric and geometric limitations imposed by the rest

of the Caskin1 structure. Caskin2 also can form oligomers through its SAM domain. Despite an approximately 60% sequence identity with the Caskin1 SAM tandem, the crystal structure of the Caskin2 SAM tandem reveals a distinct oligomeric architecture. Each SAM domain features complementary head and tail surfaces, enabling the tandem to self-interact. In the case of Caskin1, this interaction forms a compact monomer, where the two SAM domains create a tight unit. Unoccupied head and tail surfaces on these monomers allow them to link in both directions, resulting in the formation of long, linear fibrils. In contrast, the Caskin2 SAM tandem forms a domain-swapped dimer, where the SAM1 domain of one protein interacts with the SAM2 domain of another, and vice versa. Each SAM domain within this dimer retains an available interaction surface, giving the Caskin2 SAM oligomer the potential to assemble into a branched structure, unlike the linear assembly observed for Caskin1. Interestingly, when both Caskin1 and 2 tandem SAM domains were expressed in Neuro2a cells, Caskin2 was detected in punctae, while Caskin1 formed as the previously described long fibres (Wang et al., 2024, Smirnova et al., 2016, Stafford et al., 2011)

Apart from the well-organized domains on the N terminal side of Caskins, the C terminal side contains a large, unorganized, proline rich region. Intrinsic disordered regions are frequently associated with signalling and regulatory functions, due to protein–protein interactions. One commonly cited benefit of disordered proteins is that their extended structure allows for a high interaction capacity despite their relatively small size, a characteristic that may be particularly relevant for scaffold proteins. Indeed, the disordered region of Caskin1 has been shown to interact with proteins using its disordered region (Balázs et al., 2009). The C terminal part of Caskin2 seems to be a binding partner to talin, which helps to bridge the gap between integrins and the actin network and implying that Caskin2 may be an important regulator of cell motility (Goult et al., 2018, Wang et al., 2024).

Scaffold proteins such as Caskins have been proposed to play important roles in many neurodevelopmental and neuropsychiatric diseases. Caskin1 was found to be dysregulated in black and tan brachyury (BTBR) mice, a frequently used model for autism spectrum disorder (Daimon et al., 2015). A mutated variant of the CASKIN1 gene, having a missense mutation in its proline rich region, was found to segregate well with mental disorders in a family through three generations (Wahbeh et al., 2023). Thus, Caskin proteins might have relevance in neuronal diseases and cognitive functions.

Neuronal pathologies related to PSD scaffold protein dysfunctions

Psychiatric disorders, also referred to as mental disorders or mental illnesses, are health conditions characterized by significant changes in cognition, emotion and/or behaviour, often accompanied by impaired social communication and interaction. These disorders include neurodevelopmental and neuropsychiatric conditions such as autism spectrum disorder (ASD), schizophrenia, attention-deficit hyperactivity disorder (ADHD), Tourette's syndrome, obsessive-compulsive disorder, bipolar disorder, and anxiety disorders. Symptoms of ASD typically manifest in early childhood, often within the first year of life. As the term "spectrum" implies, ASD encompasses a broad range of symptoms and with varying severity, including conditions such as autism, Asperger's syndrome, and Rett syndrome. ASD is marked by restricted and repetitive behavioural patterns, along with difficulties in social communication and interaction. Each child with ASD presents a unique combination of symptoms, with varying levels of severity from mild to very severe for each trait. This variability makes ASD challenging to study, diagnose, and treat (Jung and Park, 2022).

Although *Shank* genes share a high degree of sequence and domain homology, their proteins and isoforms are expressed differently in the central nervous system depending on developmental stage, cell type, and brain region. Consequently, the neurobiological roles of SHANK proteins in the brain are complex and may vary in their involvement in neuronal and synaptic activities and functions. In recent years, distinct variations in *Shank* genes have been identified as significantly associated with the risk of various disorders (Wan et al., 2022).

Shank genes were first linked to neurodevelopmental disorders through studies of Phelan–McDermid syndrome (PMS), a condition caused by a 22q13.3 deletion. PMS is characterized by autistic-like behaviours, hypotonia, and delayed or absent speech. The SHANK3 gene is deleted in nearly all reported cases of PMS, and its loss is believed to underlie the core neurodevelopmental and behavioural deficits observed in patients. In addition, numerous SHANK3 mutations have been identified in genetic screenings of those patients with autism spectrum disorder (ASD) who do not have a PMS diagnosis, as well (Monteiro and Feng, 2017). Furthermore, SHANK3-deficient rodent models exhibit behavioural phenotypes such as increased repetitive behaviours, abnormal social interactions, heightened anxiety, impaired neuronal physiology, and altered PSD levels of HOMER, PSD-95, DLGAPs, NMDARs, AMPARs, and other proteins. These phenotypes resemble certain features of human neuropsychiatric disorders. Importantly, a meta-analysis estimated that truncating mutations in

the SHANK gene family account for approximately 1% of all ASD cases (Delling and Boeckers, 2021).

PSD-95 plays a critical role in postsynaptic development and function. During development, PSD-95 protein levels increase until late adolescence and early adulthood, aligning with the growth of synaptic density in the human prefrontal cortex. The deficiency in PSD-95 was reported to be responsible for intellectual disability, global developmental delay, autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), epilepsy, hypotonia, and movement disorders. Truncated variants representing loss-of-function proteins established a causal link between PSD-95 haploinsufficiency and neurodevelopmental and neuropsychiatric disorders. Additionally, six missense variants, all localized within highly conserved functional domains of PSD-95, have been reported to be likely pathogenic (Levy et al., 2022).

PSD-95 is known to be notably vulnerable in patients with schizophrenia (SCZ) and may contribute to disrupted spine growth and altered synaptic physiology. Neuroanatomical evidence from SCZ patients revealed structural abnormalities in specific brain regions, including a general decrease in brain volume and reduced cortical grey matter in the dorsolateral prefrontal cortex, superior temporal gyrus, and limbic regions such as the hippocampal formation and anterior cingulate cortex. At the cellular level, studies reported reductions in neuronal numbers and dendritic spine densities, particularly in the hippocampus and dorsolateral prefrontal cortex (Coley and Gao, 2017).

CASK-related disorders arise from pathogenic variants in the CASK gene and encompass a spectrum of clinical phenotypes. These disorders are broadly categorized into two primary phenotypes: MICPCH (microcephaly with pontine and cerebellar hypoplasia) and XLID (X-linked intellectual disability) with or without nystagmus. Typically, MICPCH is linked to loss-of-function mutations in the CASK gene, such as nonsense or frameshift mutations, while XLID with or without nystagmus is associated with hypomorphic mutations, such as missense variants (Mori et al., 2023). The physiological significance of CASK has been explored using ‘knockdown’ (KD) and knockout (KO) mouse models. In KD mice, where CASK expression was reduced by 70%, the animals were viable but smaller than their wild-type counterparts. In contrast, KO mice, where CASK expression was eliminated, did not survive beyond the first day after birth. Although most of the identified subcellular alterations were identified within the presynaptic region, postsynaptic consequences of CASK-related disorders cannot be ruled out completely (Liu et al., 2024).

Previous observations in Caskin-deficient transgenic mice

Our research group investigated the effects of Caskin isoforms in knockout animals, as well as using *in vitro* cultured hippocampal neurons prepared from embryos lacking both Caskin isoforms. Caskin1 and Caskin2 KO animals were generated with the help of a Cre recombinase-based system, by deleting exons 2-6 of the *CASKIN1* gene, as well as exons 3-7 of the *CASKIN2* gene, as described in Bencsik et al., 2019. In order to completely rule out Caskin isoforms dependent functions, double knockout mice possessing Caskin-related deletions in chromosome 17 and 11 were also generated and named as Caskin double knockout (dKO) mice (**Figure 4**).

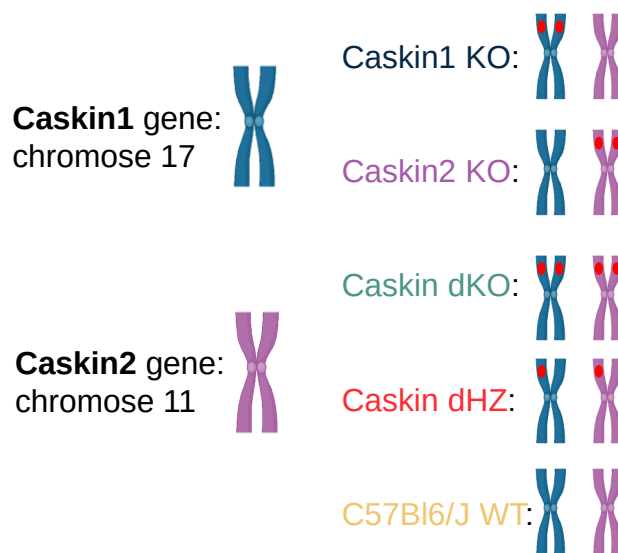


Figure 4. Indication of different genotypes employed in experiments performed on Caskin deficient mice. Deleted Caskin alleles are indicated by red dots within the chromosomes.

To investigate the effects of Caskin proteins *in vitro* and *in vivo*, multiple systems were employed. Dissociated hippocampal cell cultures lacking both Caskin isoforms revealed that dendritic spine number was reduced, and the number of filamentous, less stable forms were more prevalent. These observations indicate that Caskin proteins have a role in regulating the size and morphology of dendritic spines in neurons. It is noteworthy that Caskin1 overexpression did not rescue this phenotype. In lysates taken from wild-type cultures, Caskin1 co-precipitated with Shank2, indicating a potential interaction between endogenous Caskin1 and Shank2. There was also a sharp increase in the pS845 phosphorylation of GluA1 subunits. Caskin deficiency reduced synaptic efficacy in acute brain slices under normal conditions and during LTP formation, and lack of Caskin proteins resulted in reduced size of synaptic profiles within the CA1 hippocampal region. Taken together, our findings prove that Caskin proteins likely regulate postsynaptic organization and composition *in vivo* (Bencsik et al., 2019).

In addition, lack of Caskin proteins lead to significant behavioural changes. Caskin1 KO mice, lacking only the Caskin1 isoform, were compared to wild-type animals as control (Katano et al., 2018). Caskin1 KO animals showed significantly elevated anxiety-like behaviour in the light/dark transition tests. In the elevated plus maze test, however, Caskin1 KO animals entered more frequently into the open arms, moved longer distance and performed elevated vertical activity within the open field test. Possible social interaction deficiencies were only detected in the home cage social interaction tests, but not in the novel environment or Crawley's social interaction tests. Depressive and schizophrenia related behaviours were studied using the Porsolt forced swim test, where the KO animals showed reduced mobility. On the other hand, no differences were found in the tail suspension and startle response/prepulse inhibition tests. These results show a possible involvement of Caskin1 in a variety of disorders, from anxiety to depression.

In our lab, Caskin double knockout mice were examined, which lack both isoforms of the Caskin protein. Here Caskin double heterozygous (dHZ) animals, having one working allele for both Caskin gene, served as controls. Caskin dKO animals showed reduced learning abilities, both in a novel-object recognition test, which examines hippocampus independent memory, and in hippocampus dependent spatial memory in Morris water maze experiments. Interestingly, open field test yielded no differences between dKO and dHZ mice (Katano et al., 2018, Bencsik et al., 2019) (**Table 1**).

Animals	Behavioural tests	Results
Caskin1 KO mice, compared to wild-type controls (Katano et al., 2018)	Light/dark transition test	Distance travelled and time spent in the light chamber decreased
	Elevated plus maze	Distance travelled and number of entries into the open arms increased
	Open field test	Changes in distance moved and vertical activity
	Social interaction test in novel environment	No change detected
	Crawley's social interaction test	No change detected
	Home cage social interaction test	Activity levels were significantly reduced
	Porsolt forced swim test	Reduced mobility
	Tail suspension test	No change detected
	Startle response/prepulse inhibition	No change detected
Caskin double KO mice, compared to double heterozygote controls (Bencsik et al., 2019)	Novel object recognition test	Time spent sniffing the novel object did not differ from the familiar object
	Open field test	No change detected
	Morris water maze	The time and distance before reaching the platform increased

Table 1. Overview of the relevant behavioural analysis performed so far on Caskin deficient mice.

Aims of the project

As previous experiments had somewhat contradicting results obtained from Caskin1 or Caskin double knockout mice, the importance of Caskin1 and/or Caskin2 isoforms in the observed behavioural or structural changes could not been determined. Additionally, isoform-specific alterations of AMPA receptor expression and intracellular trafficking may contribute to dysfunction in neurodevelopmental disorders such as ASD (Bencsik et al., 2023). With these in mind, our main aims were to

- Investigate Caskin isoform specific effects on the marble burying behaviour of transgenic mice;
- Analyse the subcellular localization of AMPA receptor GluA1 subunits within the medial prefrontal cortex.

Materials and Methods

Animals

Our transgenic mice were housed in the animal facility, at 22 ± 1 °C, with 12-hour light-dark cycles, and an *ad libitum* access to food and water. The animal lines were: Caskin double knockout (Caskin dKO) mice lacking both isoforms of the Caskin proteins, Caskin1 knockout (Caskin1 KO), which lacks the Caskin1 isoform but expresses Caskin2; Caskin2 knockout (Caskin2 KO), which lacks the Caskin2 isoform but expresses Caskin1. Caskin double heterozygotes (Caskin dHZ, possessing one functioning and one non-functioning allele for both isoform) and C57Bl6/J (WT or wild-type) mice were used as controls. To obtain Caskin1 and Caskin2 KO animals, as well as heterozygotes, we bred Caskin dKO animals with wild-types and then performed a PCR to confirm the correct genotype. Separation of the animals, as well as regular bedding, water and food changes were performed by the person conducting the experiments to minimize any interference. Animals were maintained and handled in accordance with the Guidelines for Accommodation and Care of Animals, according to the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. This study was conducted under the approval of the Institutional Animal Ethics Committee of Eötvös Loránd University (approval number: PEI/001/1108-4/2013 and PEI/001/1109-4/2013). All methods were performed in accordance with the guidelines on research ethics of Eötvös Loránd University.

Marble burying test

The tests were executed in a separate room from the normal animal housing. Mice were transported to this room at least one week before the test. Only male mice were tested, which were kept together with their littermates, and which have never been mated. The tests were carried out at 2, 4 and 6 months of age in a 25 cm x 42 cm non-transparent cage. Twenty-four, 15mm wide transparent glass marbles were placed in the cage. Fresh bedding and clean cage and marbles were used in every test, to remove possible olfactory cues. The bottom of the cage had 5 cm of the same bedding what the animals had in their home cage. The animals were placed in the cage for 30 minutes, during which time their activity was recorded with a camera in full HD with 60 fps (Sjcam Sj4000). After the test, animals were moved back to their home cage. During the evaluation, any marble which was more than 50% covered by the bedding was counted as “buried”. The recordings were evaluated using an automated ImageJ plugin, AnimalTracker (Gulyás et al., 2016) by determining the distance moved and the total amount of the mobility and the immobile time. Using a stopwatch, the total time spent with interacting with the marbles (e.g., sniffing, burying or tossing the marbles) was also measured.

Immunohistochemistry

Caskin double knockout (Caskin dKO) and wild-type (C57Bl6/J wild-type) animals were deeply anesthetized using Isoflurane. Transcardial perfusion was performed using cold PBS followed by 4% paraformaldehyde (TAAB, wt/vol in PBS; pH 7.4). The brain was removed and postfixed in 4% PFA dissolved in PBS overnight, than placed in 30% sucrose in PBS. Using a cryostat (Leica), 50 µm thick coronal slices were made containing the medial prefrontal area and amygdala and placed into PBS solution. 0.5% Triton X-100 in PBS was used to permeabilize the slices. 2 % BSA + 0.1% Triton X-100 + 0.1% sodium-azide solution dissolved in PBS was used for blocking the non-specific binding sites. Primary antibodies were dissolved in the blocking solution, then applied overnight in 1:500 concentration, at 4 °C (anti-GluA1, rabbit, Abcam; anti-βIII-tubulin, mouse, EXBIO Praha). Secondary antibodies were applied in 1:300 dilution in blocking solution for 90 minutes (goat anti-rabbit IgG (H+L) labelled with Alexa Fluor™ 546, ThermoFisher; goat anti-mouse IgG (H+L) Alexa Fluor™ 488, ThermoFisher). Slices were mounted on poly-L-lysine (Sigma-Aldrich) coated glass slides and mounted with 4-88 Mowiol (Sigma-Aldrich). Tile images were obtained with fluorescent microscopy, using a Axio Observer Z1 epifluorescent microscope (Carl Zeiss), with a Zeiss 20x Plan-Neofluar objective (Numerical Aperture: 0.4). Higher magnification images were taken

with an LSM 800 (Carl Zeiss) microscope with Plan Apochromat 63x (Numerical Aperture: 1.4) oil-immersion objective. Confocal images were taken by an LSM 800 laser point scanning microscope using 405 nm (DAPI), 488 nm (β III-tubulin) and 561 nm (GluA1) laser lines.

Statistics

Statistics were performed using the IBM SPSS software version 29.0.0.0. Shapiro–Wilk test was used to check the normality of the data, but we found that most of it did not have a normal distribution. Mann-Whitney test was used as a non-parametric, independent sample test.

Results

Lack of Caskin proteins induces changes in marble burying behaviour

In rodents, repetitive behaviours are common and include activities such as grooming and digging. Such repetitive behaviours can be examined by paradigms such as the marble burying test (Thomas et al., 2009). The marble burying test leverages the natural tendency of mice to dig, as they would in natural settings such as burrows or escape tunnels, and in standard cage bedding (Angoa-Pérez et al., 2013). This method offers several advantages, including its simplicity, the accuracy of behavioural scoring, the spontaneous nature of these behaviours in rodents, and their suitability for high-throughput screening of genetically modified mice for abnormal behavioural phenotypes.

In order to understand the behavioural differences caused by deleting Caskin1 and/or Caskin2 isoforms, five different animal strains were used. Besides the Caskin dKO line, where both Caskin isoforms are deleted (generated as described in Bencsik et al., 2019), we generated two new strains, having either two alleles of Caskin1 or Caskin2 proteins while lacking the other isoform (designated as Caskin2 KO and Caskin1 KO mice, respectively). It is important to note that as the genes for the two Caskin isoforms are located on two different chromosomes, technically it was not possible to use littermates of the single or double KO mice as controls. Thus, we used two additional mouse strains as controls: either double heterozygotes expressing only one functional allele of Caskin1 and Caskin2 (dHZ mice) or C57Bl6/J mice as wild-type controls (see **Figure 4**). Genotypes were confirmed using PCR genotyping (data not shown).

First, we analysed the distance moved by the animals during the 30 min-long observation time as described in the methods section (**Figure 5**). At two months of age, slight differences were detected between how much the animals moved within the cage. At later time points, however, this difference seemed to disappear, and it is important to note that if we remove some extreme outliers, the significant differences will be abolished. Thus, deletion of either a single or both Caskin isoforms did not have strong effects on the motor activity of the transgenic mouse strains.

Next, we compared the number of buried marbles as an end-point readout of the burying activity at 2, 4 and 6 months of age (**Figure 6**). Firstly, it is interesting to note that the two control groups, the wild-type (yellow bars) and double heterozygote (dHZ; red bars) animals differed significantly at all three examined timepoints as dHZ buried more marbles. This means that heterozygotes seem to be better for comparison with the other KO lines. It also points to

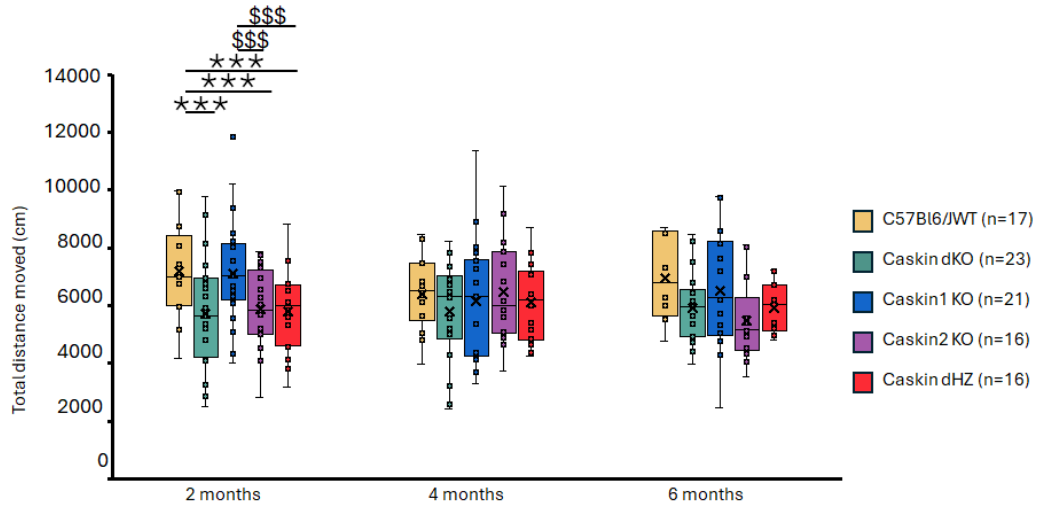


Figure 5. Distance moved within the 30 min-long observation time as determined by an automated ImageJ plugin from recordings obtained from different Caskin mouse strains at different ages. Significance levels: *: $p < 0,05$, **: $p < 0,001$, ***: $p < 0,0001$, \$: $p < 0,05$, \$\$: $p < 0,001$, \$\$\$: $p < 0,0001$. *: differences to the control \$: differences between mutant genotypes.

the fact that Caskin proteins seem to have a dose-dependent effect on burying activity. It is also important to note that neither of these control lines show age-dependent changes in the number of buried marbles (**Figure 6**).

Caskin2 KO animals performed similar activity compared to heterozygote animals and they differed from wild-type animals only at the earliest timepoint, at 2 months of age. Similarly to WT and dHZ mice, Caskin2 KO animals did not show age-dependent changes in burying behaviour (**Figure 6**; purple bars).

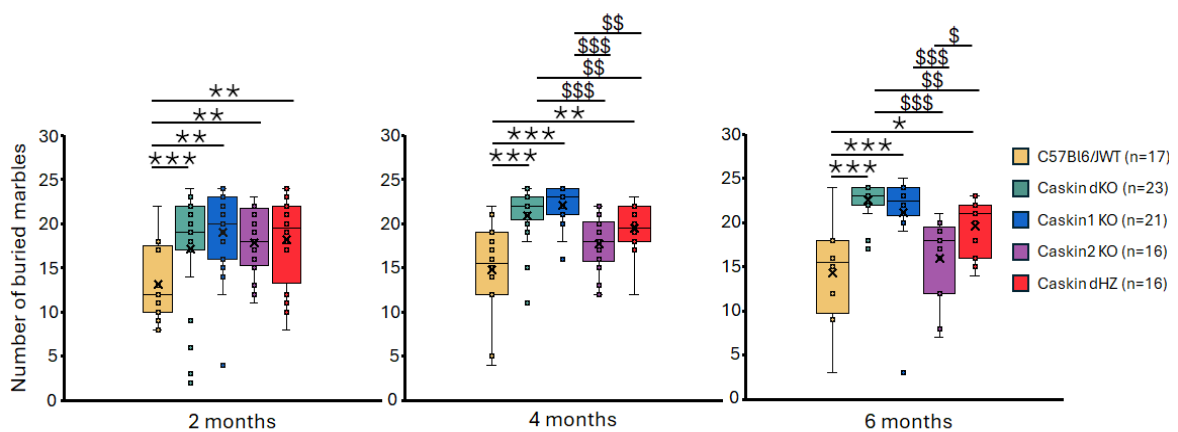


Figure 6. The number of buried marbles at different ages grouped by genotype. Significant differences compared to the wild-type and other mutants are indicated with the horizontal lines. Significance levels: *: $p < 0,05$, **: $p < 0,001$, ***: $p < 0,0001$, \$: $p < 0,05$, \$\$: $p < 0,001$, \$\$\$: $p < 0,0001$. *: differences to the control \$: differences between mutant genotypes

Importantly, Caskin1 KO (blue bars) and Caskin dKO (green bars) animals performed very similarly. Firstly, both strains showed increased burying behaviour compared to WT and Caskin dHZ animals. At four and six months of age, their burying behaviour was elevated compared to Caskin2 KO animals, as well. Both strains showed an age dependent increase in the number of buried marbles, reaching near-maximum values by 4 and 6 months of age (**Figure 6**).

Taken together, these results show that Caskin genes have a dose dependent effect associated with them, and that Caskin1 and Caskin2 isoforms have isoform-specific effects on the marble burying behavioural phenotype.

Total interaction time reveals a Caskin1-mediated effect on marble burying behaviour

Counting the number of buried marbles only gives us a snapshot of the endpoint of the test. In addition, Caskin1 and Caskin dKO animals showed such a high burying activity that we could not discern possible differences in phenotype because the animals buried all marbles. Thus, we further investigated the recordings by determining the time for how long each animal interacted with the marbles. This included digging behaviour, as well as sniffing and nudging of the marbles. The total interaction time was calculated in seconds and then were compared. At the time of writing this work, data are available only from the 4- and 6-months old groups while the 2-month-old recordings are still under evaluations. It is also important to note that at the current stage, we have much less data points in this evaluation (10 vs. 16-23 for the previous essays), thus the data have large variance (**Figure 7**).

According to our current data, at four months of age, Caskin2 KO animals have very similar interaction times compared to the heterozygotes but differ from the wild-types. At six months of age, on the other hand, Caskin2 KO mice possessed less interaction compared to Caskin dHZ animals but did not differ from WT animals. In accordance with previous analyses, Caskin1 and dKO animals showed similarly high interest towards the marbles. In case of the Caskin1 KO animals, we can see larger amount of variance, while Caskin dKO animals almost all present with very high interaction times. These observations already indicate that the lack of Caskin1 isoform specifically leads to increased interest towards the marbles (**Figure 7**). Nevertheless, further analysis is in progress regarding the two months old animals, and also to increase the number of data points in hopes of reducing the variance of the data points to more clearly identify differences.

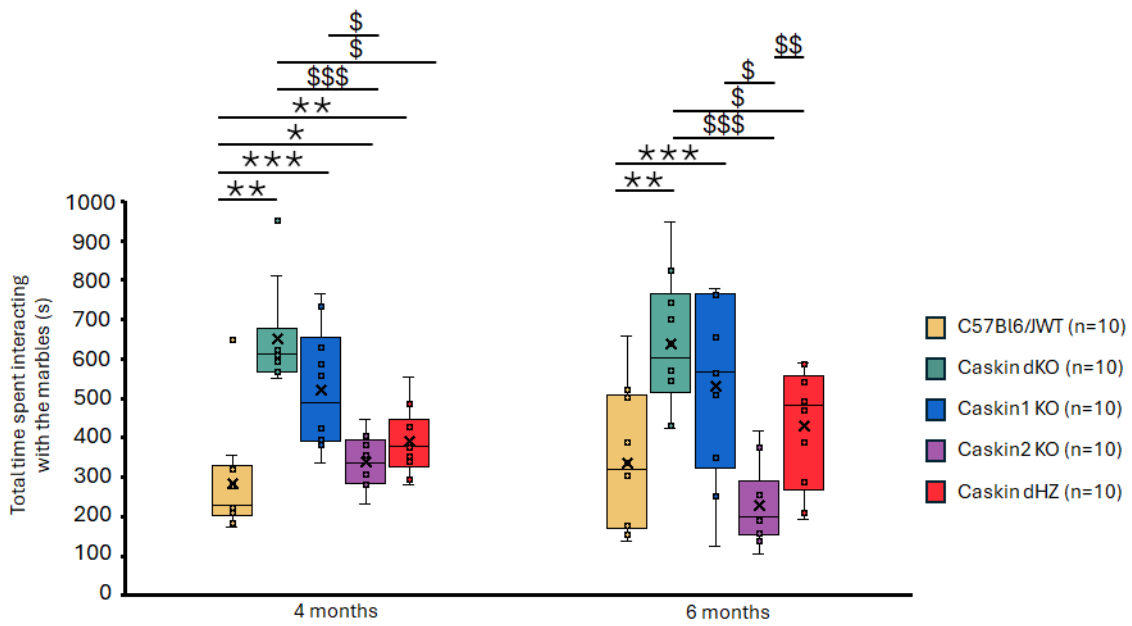


Figure 7. Evaluation of the total time animals spent interacting with the marbles. Significance levels: *: $p < 0,05$, **: $p < 0,001$, ***: $p < 0,0001$, \$: $p < 0,05$, \$\$: $p < 0,001$, \$\$\$: $p < 0,0001$. *: differences to the control \$: differences between mutant genotypes.

Lack of Caskin proteins induces potential changes in the amount of GluA1 subunits of AMPA receptors in the medial prefrontal cortex

The mPFC (medial prefrontal cortex) is a cortical region which plays a crucial role in regulating emotion, motivation and sociability. The mPFC has an important connection with the amygdala, and this connection is implicated as an important regulator of social responses in rodents, monkeys and humans (Meisner et al., 2022). The mPFC can be subdivided into the anterior cingulate cortex (ACC), the prelimbic (PrL) and the infralimbic cortex (IL) (Bloem et al., 2014). We picked the prelimbic cortex for our current investigation.

Slices taken from the prefrontal cortex of Caskin dKO and wild-type mature animals were immunostained using AMPA receptor GluA1 subunit specific antibodies and β III-tubulin antibodies in combination with DAPI to label nuclei (**Figure 8** and **9**). Confocal microscopy images were taken from 2nd/3rd layer neurons (see the dense nuclear staining on **Figure 8A**). According to our preliminary results, large structural changes between WT and Caskin dKO brain sections were not found. β III-tubulin signal clearly labelled the apical dendrites running parallel with each other, perpendicular to the pial surface, while basal dendrites were identified as running oblique to the apical ones around the cell bodies (**Figure 8/B**).

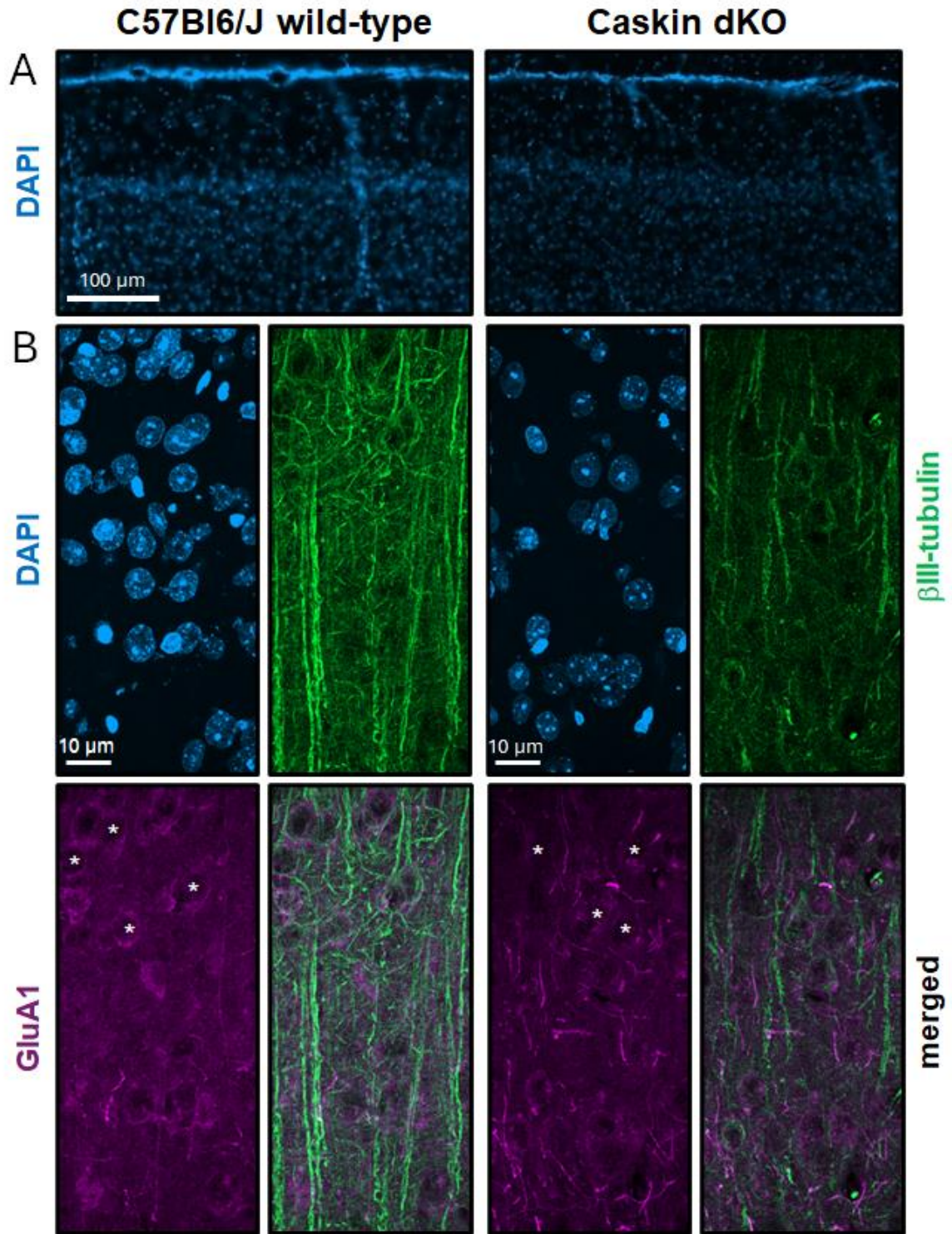


Figure 8. Images taken from the medial prefrontal cortex of C57Bl6/J wild-type and Caskin dKO mice. A: Representative images of DAPI staining in the prelimbic area of the medial prefrontal cortex. No large structural differences can be observed. B: DAPI, β III-tubulin and GluA1 signals in the prelimbic cortex. Stars indicate cell bodies. Scale bar: 100 μ m and 10 μ m.

When images were taken with a higher magnification, WT basal dendrites had mainly dot-like GluA1-positive puncta around them (see the depicted area from the middle column, enlarged within the right column in **Figure 9**). Interestingly, within the basal dendrites of Caskin dKO slices, GluA1 immunoreactivity was the strongest within the dendrites.

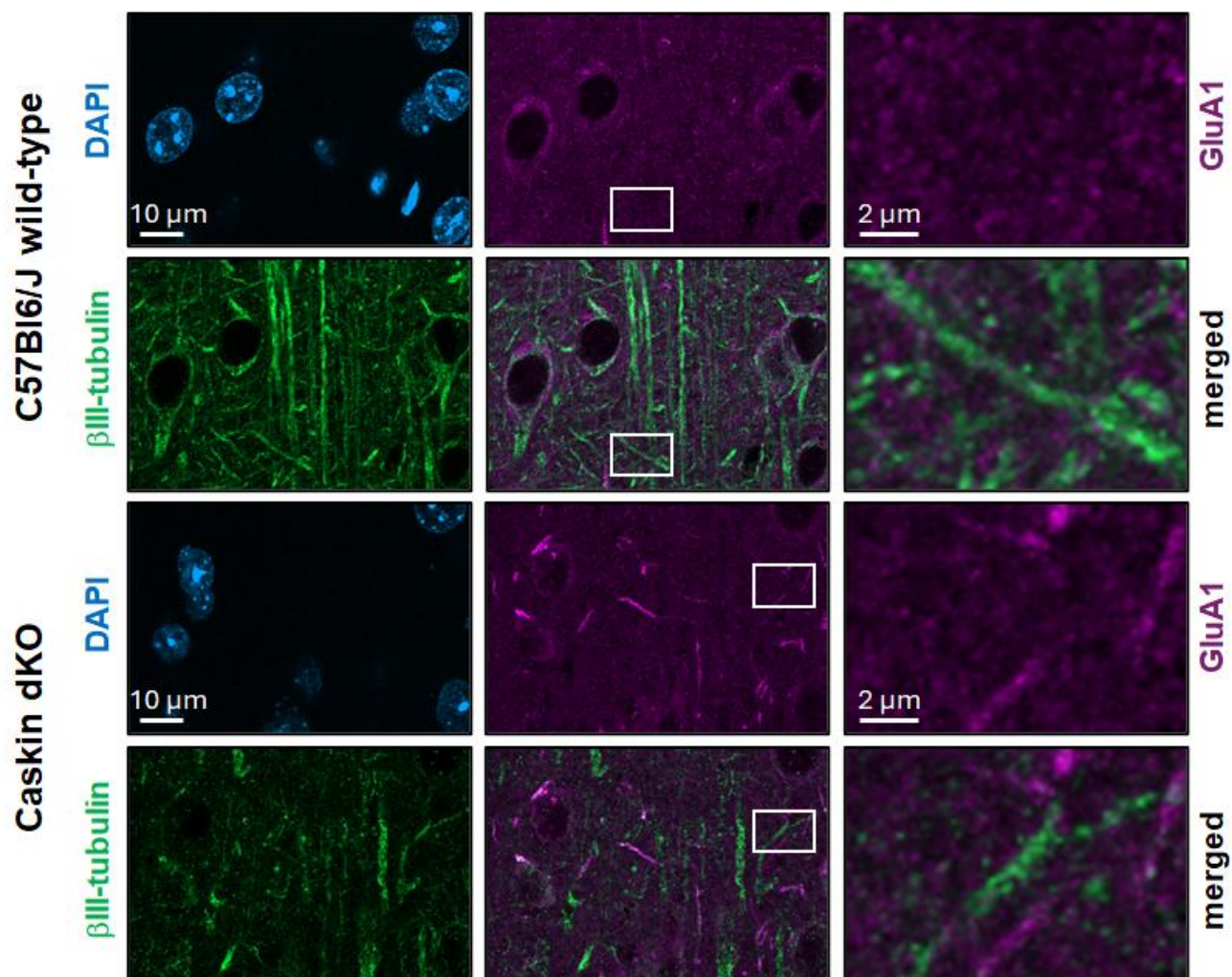


Figure 9. Images showing layer 2nd/3rd neurons in the prelimbic area, with enlarged images showing the GluA1 signal in the basal dendrites. Scale bar: 10 μm.

Summary of the results

During my TDK work, I was able to show that:

- The results taken from the double heterozygote animals show a dose dependent effect of Caskin isoforms on marble burying.
- Deletion of the two Caskin isoforms have different behavioural effects, implying that the lack of Caskin1 isoform selectively lead to increased burying activity.
- Our preliminary immunohistochemistry experiments show an increase in the GluA1 immunoreactivity in the pyramidal neurons of the medial prefrontal cortex, and an accumulation of GluA1 subunits in the basal dendrites of the 2nd/3rd layer cells.

Discussion

During my TDK work, we showed that the two isoforms of the Caskin protein, Caskin1 and Caskin2 have isoform-specific effects on behaviour. While the knockout of Caskin1 isoform seems to induce severe repetitive behaviours, deletion of Caskin2 isoform seems to have little - or indeed, opposite - effect, based on the decreased marble burying activity of the animals. Our preliminary results also indicate that upon the deletion of both Caskin isoforms, the intracellular localisation of AMPA receptor subunit GluA1 seems to change, indicating impaired delivery to the postsynaptic membrane. These conclusions, however, need additional investigations and further work is required to confirm these findings. It is also required to see whether slices taken from Caskin1 KO or Caskin2 KO animals show similar results as the ones from dKO animals, especially when performing a more extensive regional analysis and cell-type specific characterisation of GluA1 intracellular localisation.

Nevertheless, we consider these results significant as to our best knowledge, our work is the first to directly compare the behavioural consequences of deleting only one or both Caskin isoforms. While scaffold proteins are subject to large amount of research due to their implicated roles in human neuropsychiatric and neurodevelopmental diseases, little is known about the functional similarities or redundancy between the two Caskin isoforms (Soler et al., 2018).

Multiple lines of Shank3 mutant animals, including mice, rats, and macaques with global, conditional, point mutations, or knockout (KO) of the Shank3 gene, have been developed. Commonly identified changes include anxiety-like behaviours, abnormal locomotor activity, and impairments in learning and memory. Interestingly, Shank isoform deficient animals show reduced burying behaviours (Jaramillo et al., 2017, Garrido et al., 2022), in contrast to our results presented here, where Caskin1 KO and Caskin dKO animals buried more and spent more time with the marbles. Additional behavioural parameters, such as ultrasonic vocalizations and learning and memory, have been also assessed in various Shank3 mutant lines, yielding distinct results. These differences may reflect isoform-specific disruptions in each line. The concept that isoform-specific disruptions lead to distinct phenotypic outcomes—and potentially to different disorders—is definitely important (Zhou et al., 2016). This variability highlights the complexity of Shank3-related phenotypes and supports a wider framework of isoform specific effects of scaffold proteins, including Caskins (Wan et al., 2022, Ferhat et al., 2023).

Parallel to our marble burying test, self-grooming tests were also performed at our lab (non-published results). This test is used to study repetitive behaviour in rodents, and exploits

their natural behaviour of self-grooming, which is one of the most common behaviours in mice (Kalueff et al., 2016). Studying the effect of neuronal scaffold protein knockdown on this behaviour was already done in Shank3 mutant mice, where excessive self-grooming was observed (Ferhat et al., 2023). According to our non-published results, transgenic mice lacking Caskin isoforms show reduced grooming behaviour, with Caskin2 seemingly playing a more prominent role. We also performed elevated plus maze experiments, which is widely used to study anxiety in rodents and exploits their tendency to prefer enclosed spaces (Komada et al., 2008). Our preliminary observations show no differences between the Caskin genotypes but as already emphasized, further investigations are needed to clarify the behavioural consequences of deleting or reducing Caskin isoform levels.

The dose-dependent effect what we observed when comparing wild-type and double heterozygous animals is interesting in the light of the fact that multiple scaffold proteins, such as PSD-95 (Horner et al., 2018) and Shank3 (Bozdagi et al., 2010) have been identified to have similar effect. Due to the limitation of the double heterozygote model used in our experiments, we cannot currently decide which of the two genes, or both genes causes the dose-dependency. Based on our marble burying tests, however, it is likely Caskin1. Such isoform dependent effect is also supported by other scaffold protein related research, for example the Shank1 isoform of the Shank protein seems to carry less significance of dose compared to Shank3 (Wang et al., 2016)

Interestingly, neurodevelopmental disorders risk genes have been linked to synaptic structure and function. MRI data suggests that disrupted neuronal networks may exist before the onset of behavioural symptoms in children with autism. One of the most prevalent explanations for many of these disorders is an impairment of the normal excitation/inhibition ratio. Dysregulation of AMPAR trafficking is a possible factor contributing to E/I imbalances. Monogenic neurodevelopmental disorders with autism-like symptoms, such as fragile X syndrome and Rett syndrome, are also associated with AMPA receptor impairments. However, most individuals with autism exhibit a range of de novo low-penetrance genetic mutations in genes regulating AMPA receptor trafficking (Bencsik et al., 2023, Jimenez-Gomez et al., 2024). Indeed, it is very likely that Caskin protein knockdown also interferes with AMPA receptor subunit GluA1 trafficking.

Scaffold proteins play a huge role in regulating the surface expression of AMPA receptors, either through trafficking or anchoring. Caskins' main binding partner, CASK for example, is known to regulate SAP97 conformation, and through that its binding to either

AMPA or NMDA receptors (Lin et al., 2013). The CASK-Mint1-Velis-SAP97 pathway preferentially targets specific receptors to the membrane, and Caskin is known to compete with Mint1 for CASK binding, meaning that it can be a possible regulatory step in the selective targeting of different receptors to the membrane (Tabuchi et al., 2002; Leonoudakis et al., 2004).

At the moment, our immunohistochemistry work is limited by the small number of experiments performed until this time. It would be highly advantageous to pair our current immunostainings with additional selective markers, such as parvalbumin, CTIP2 or calbindin (Hevner, 2007) to be better able to identify the exact type of neurons examined within the mPFC. Other AMPA subunits, for example subunit GluA2 should also be investigated, as they also play roles in a variety of function, and are heavily implicated in disease (Salpietro et al., 2019).

There is also a need for additional experiments to further confirm our findings, and we are looking to expand immunostaining for other genotypes such as Caskin double heterozygotes and Caskin1 and 2 KO animals. This would allow us to make a more solid theory regarding the role these proteins play in the trafficking of the different AMPA receptors.

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