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**Characterization of *Trpv1*-expressing neurons in the
mouse supramammillary nucleus**

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Abstract

It is well known that the transient receptor potential vanilloid 1 (TRPV1) ion channel is predominantly expressed in primary sensory neurons located in the dorsal root and trigeminal sensory ganglia. This non-selective cation channel plays a significant role in pain and neurogenic inflammation. *Trpv1* expression was also demonstrated in the central nervous system (CNS), but much less is known about the expression pattern of this capsaicin-sensitive receptor-ion channel within the brain. Therefore, we aimed to elucidate *Trpv1* expression in the mouse CNS. Since a specific anti-TRPV1 antibody is not available, *Trpv1* mRNA expression in coronal sections of the mouse brain was detected with RNAscope *in situ* hybridization technology. In addition, we co-localized *Trpv1* mRNA with specific neuronal markers, such as *Vglut2* (vesicular glutamate transporter 2) mRNA for glutamatergic and tyrosine hydroxylase immunopositivity for dopaminergic neurons. We found that *Trpv1* mRNA is selectively expressed in the mouse supramammillary nucleus (SuM). Within the mouse SuM, we found a strong co-expression of *Trpv1* and *Vglut2* mRNA, but no overlap between *Trpv1* mRNA and tyrosine hydroxylase signal. Our results confirmed that *Trpv1* mRNA is specifically expressed on *Vglut2*-positive glutamatergic neurons in the mouse SuM. We propose that our finding may serve as the basis for narrowing down the scope of investigation of the operation and function of the TRPV1 channel in this brain region.

Introduction

1. TRPV1 receptor – ion channel

Transient receptor potential vanilloid 1 (TRPV1) is a member of the transient receptor potential (TRP) family (Vennekens et al., 2012). TRPV1 is a non-selective cation channel, which displays higher permeability to divalent cations, such as Ca^{2+} and Mg^{2+} , than to monovalent cations, such as Na^+ (Ahern et al., 2005; Michael J. Caterina et al., 1997; Mohapatra & Nau, 2003). TRPV1 is among the most intensively studied mammalian Transient receptor potential (TRP) channels (Stuart Bevan et al., 2014).

1.1 Structure and sensitivity

TRPV1 consists of six transmembrane (TM) segments and intracellularly located N- and C- termini (Stuart Bevan et al., 2014). The central pore containing an outer pore ‘turret’, a pore helix, and a selectivity filter is located at the pore-forming loop between TM segments 5 and 6 (Latorre et al., 2009). Binding sites of several substances, which can induce agonistic responses have been identified at the pore loop.

Additionally, both termini are found to contain multiple ligand-binding and phosphorylation sites, which play important roles in regulating TRPV1 sensitivity and activity (Bhave et al., 2002; Lishko et al., 2007). Such sites can also be found along several segment-linker domains. The overall structure of TRPV1 has been illustrated in Figure 1.

TRPV1 is activated by vanilloid compounds, such as capsaicin (S. Bevan et al., 1992a). Besides, a number of other plant products can also act as TRPV1 agonists, including piperine, zingerone, gingerol and shogaol (Nilius & Appendino, 2013). In addition to plant-derived agonists, some animal-derived toxins have been shown to act on TRPV1 (Bohlen & Julius, 2012; Siemens et al., 2006). Endogenous TRPV1 agonists, such as anandamide, 12- and 15-(S) hydroperoxyeicosatetraenoic acids (HPETE), and leukotriene B4 have also been identified (Hwang et al., 2000; Zygmunt et al., 2013).

TRPV1 also displays a certain sensitivity to extracellular pH level (S. Bevan & Yeats, 1991) and heat (Cao et al., 2013).

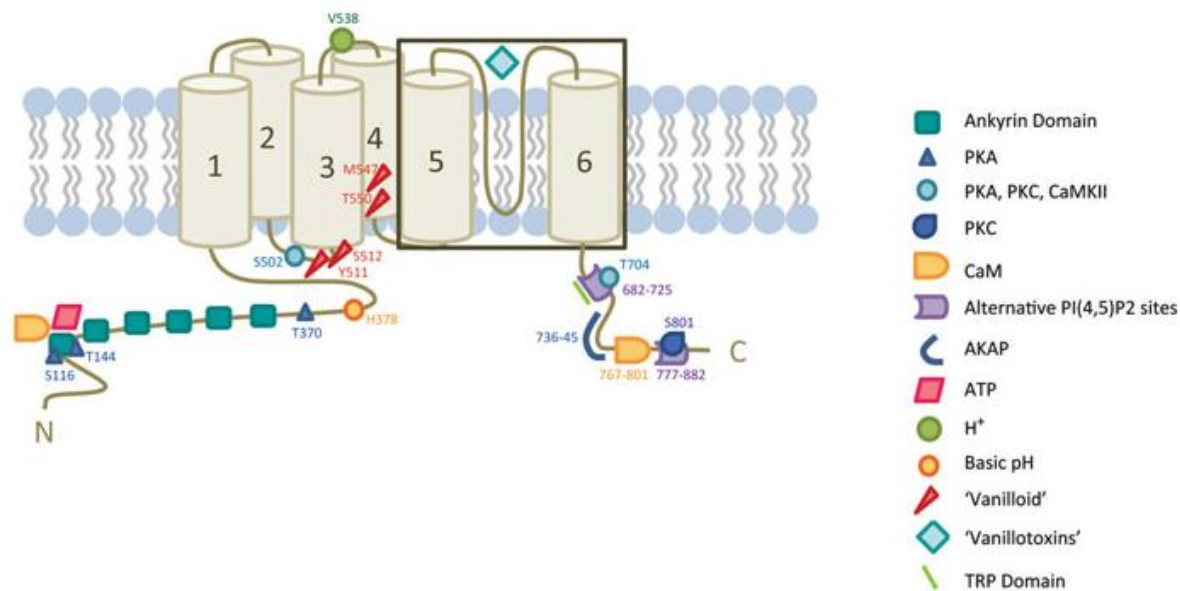


Figure 1. Key structural features of TRPV1 channel. Abbreviations: PKA, protein kinase A; PKC, protein kinase C; CaMKII, calcium-calmodulin kinase II; CaM, calmodulin; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; AKAP, A-kinase-anchoring protein (Stuart Bevan et al., 2014).

1.2 Functions

The molecular mechanism of heat and pain sensation and the physiological role of TRPV1 has been widely elucidated by David Julius who was awarded by Nobel Prize in Physiology or Medicine in 2021. (Michael J. Caterina et al., 1997; Tominaga et al., 1998). His research along with Janos Szolcsanyi's findings have revolutionized our understanding of the way how temperatures are converted into electrical signals in the nervous system (Szolcsányi, 1977).

TRPV1 is a major peripheral thermosensor in a subset of primary afferent neurons responsible for transducing thermal, chemical, and physical nociception. Activation of TRPV1 either by temperatures in noxious heat range (typically above 42 °C) or by chemical agonists, such as capsaicin, can induce sensation of 'burning' heat (Szolcsányi, 1977). While this sensation does not rely solely upon TRPV1 activation (M. J. Caterina et al., 2000b), studies have shown that antagonism of TRPV1 results in a reduction in heat sensitivity so that normally noxious temperatures might not be sensed as painful (R. Eid, 2011). Tissue injury or inflammation mediators can sensitise TRPV1 and lower the temperature threshold for activation (Immke & Gavva, 2006). This reduction leads to thermal hyperalgesia (a condition where temperatures below 42 °C can still elicit painful sensation) (Bölcskei et al., 2005; M. J. Caterina et al., 2000a).

TRPV1 has been shown to be contributed in maintaining body temperature. Administration of TRPV1 agonists induced hypothermia (Jancsó-Gábor et al., 1970a, 1970b). In contrast, hyperthermic responses can be elicited by application of TRPV1 antagonists (Gavva, Bannon, Surapaneni, et al., 2007a). Desensitization of TRPV1 was shown to impair the ability to regulate body temperature at high ambient temperatures (Jancsó-Gábor et al., 1970b). However, *Trpv1*^{-/-} mice were found to have normal core body temperatures (D. M. Tóth et al., 2011), suggesting the role of TRPV1 in thermoregulation in response to environmental changes.

Besides its well-studied role in pain sensation, recent studies indicated the involvement of TRPV1 in the transmission of pain. Intrathecal administration of TRPV1 antagonists such as AMG9810, AS1928370 or N-(4-tertiarybutylphenyl)-4-(3-chlorophenyl)-2-methyl-5-pyridinyl-tetrahydroprazine-1(2H)-carboxamide (BCTC) produced analgesic effects on several rodent models (Kanai et al., 2005; Watabiki et al., 2011; Wu et al., 2013). Under pathological states, spinal TRPV1 was demonstrated to contribute to pain exacerbation (Kim et al., 2014; Xu et al., 2010).

TRPV1 was also proposed for its role in mediating changes induced by drug treatment or stress conditions. TRPV1 antagonism was shown to lower the preference for ethanol and discourage cocaine relapse in mice (Adamczyk et al., 2012; Blednov & Harris, 2009). On the other hand, TRPV1 activation increased ethanol preference and reduced nicotine-induced depression (Blednov & Harris, 2009; Hayase, 2011). *Trpv1*^{-/-} mice exhibited fewer anxiety- and fear-induced responses (Marsch et al., 2007). These evidence suggested broad functions of TRPV1 in behavioral responses.

In addition, *Trpv1*^{-/-} mice showed impaired responses to hypertonic stimuli. It has been suggested that a variant of the TRPV1 channel is necessary for osmosensory transduction (Ciura & Bourque, 2006; Naeini et al., 2006).

It can be concluded that apart from being a conventional nociceptive sensor, TRPV1 participates in a wide range of physiological processes within the CNS. Further research into the activity of TRPV1 in the CNS would examine the mechanism in which TRPV1 is involved such processes, thus providing a different insight into the role of TRPV1 within our bodies.

1.3 Expression pattern

TRPV1 is expressed predominantly in primary sensory neurons located in the dorsal root, trigeminal and vagal ganglia (Michael J. Caterina et al., 1997; Helliwell et al., 1998). TRPV1 was also found in the central projections from sensory neurons located in the dorsal spinal cord, in the trigeminal nucleus caudalis, and the nucleus of the solitary tract (Szallasi & Blumberg, 1999; Tominaga et al., 1998).

It has been long accepted that TRPV1 is also present in the CNS. *In situ* hybridization (ISH) suggested *Trpv1* expression in the rat olfactory cortex, septal nucleus, hippocampus, locus coeruleus, substantia nigra, and the inferior olive (Mezey et al., 2000). Immunohistochemistry (IHC) further indicated the expression of TRPV1 in numerous rodent brain structures, including the hippocampus, basal ganglia, hypothalamus, thalamus, and the cerebellum (Cristino et al., 2006; Mezey et al., 2000; A. Tóth et al., 2005). Reverse transcription (RT)-PCR and Western blot analysis also suggested widespread distribution of *Trpv1*/TRPV1 in rodent CNS (Mezey et al., 2000; A. Tóth et al., 2005). Besides, pharmacological studies have demonstrated numerous capsaicin-evoked physiological and electrical responses in multiple brain regions (Hori et al., 1988; Mezey et al., 2000; Steenland et al., 2006; Szabo et al., 2002).

However, in contrast to the widespread and robust distribution of TRPV1 in the CNS proposed by previous studies, *Trpv1*-specific bacterial *lacZ* reporter gene expression results confirmed *Trpv1* expression only in few brain areas, including the intrafascicular nucleus, the periaqueductal grey matter, the supramammillary nucleus, hypothalamus, and hippocampus (Cavanaugh et al., 2011).

One must note that several publications elucidating TRPV1 expression in rodent CNS seem to lack reliable positive/negative controls in the experimental design or omit part of their results. Limits of traditional techniques should also be considered. These problems make it difficult to evaluate the trustworthiness of existing literature. Therefore, TRPV1 expression in the CNS is still a controversial topic. A list of potential *Trpv1*-expressing regions can be found in Table 1.

Region	Species	Technique	Critical evaluation	References
Hippocampus	Mouse, rat	IHC, ISH, RT-PCR, Western blot, reporter gene expression, pharmacological approaches	Given robust supporting evidence, it is very likely that TRPV1 is expressed in the hippocampus.	(Al-Hayani et al., 2001; Cavanaugh et al., 2011; Cristino et al., 2006; Mezey et al., 2000; A. Tóth et al., 2005)
Hypothalamus	Mouse, rat	IHC, ISH, RT-PCR, Reporter gene expression, pharmacological approaches	Given robust supporting evidence, it is very likely that TRPV1 is expressed in the hypothalamus.	(Cavanaugh et al., 2011; Cristino et al., 2006; Jancsó-Gábor et al., 1970b; Mezey et al., 2000)
Thalamus	Mouse, rat	IHC, ISH, pharmacological approaches	Immunohistochemical staining indicated the presence of TRPV1, but <i>Trpv1</i> reporter mice could not detect its expression. ISH results were not shown. Therefore, it is difficult to conclude whether TRPV1 is expressed in the thalamus.	(Andoh et al., 1980; Cavanaugh et al., 2011; Cristino et al., 2006; Mezey et al., 2000)

Region	Species	Technique	Critical evaluation	References
Basal ganglia	Mouse, rat	IHC	Immunohistochemical staining suggested the expression of TRPV1 in the region, but reporter gene expression driven from <i>Trpv1</i> locus did not. It is difficult to conclude whether TRPV1 is expressed in the basal ganglia.	(Cavanaugh et al., 2011; Cristino et al., 2006)
Olfactory cortex	Rat	ISH	ISH showed the expression of <i>Trpv1</i> in the region, but <i>Trpv1</i> reporter mice could not. It is difficult to determine if <i>Trpv1</i> is expressed in the olfactory cortex.	(Cavanaugh et al., 2011; Mezey et al., 2000)
Septal nucleus	Rat	IHC, ISH	ISH demonstrated the expression of TRPV1 in the region, but <i>Trpv1</i> reporter mice could not. IHC results were omitted. Therefore, it is difficult to conclude whether TRPV1 is expressed in the thalamus.	(Cavanaugh et al., 2011; Mezey et al., 2000)

Region	Species	Technique	Critical evaluation	References
Cerebellum	Mouse, rat, human	IHC, ISH, Western blot, pharmacological approaches	While <i>Trpv1</i> reporter mice could not detect <i>Trpv1</i> expression, many experiments suggested the presence of TRPV1 in the regions. TRPV1 may be expressed in the cerebellum, but it would require further investigation.	(Cavanaugh et al., 2011; Cristino et al., 2006; Mezey et al., 2000; Salt & Hill, 1980; A. Tóth et al., 2005)
Substantia nigra	Mouse, rat	IHC, ISH, pharmacological approaches	While <i>Trpv1</i> reporter mice could not detect <i>Trpv1</i> expression, many experiments suggested the presence of TRPV1 in the regions. It seems that TRPV1 is expressed in the substantia nigra, but it would require further investigation.	(Cavanaugh et al., 2011; Cristino et al., 2006; Hajós et al., 1988; Mezey et al., 2000)
Locus coeruleus	Rat	IHC, ISH, pharmacological approaches	The insufficiency of supporting studies combined with disagreement among existing literature make it difficult to determine if TRPV1 is expressed in the locus coeruleus.	(Cavanaugh et al., 2011; Jancsó-Gábor et al., 1970b; Mezey et al., 2000)

Region	Species	Technique	Critical evaluation	References
Inferior olive	Mouse, Rat	IHC, ISH	While IHC and ISH results showed TRPV1 expression, <i>Trpv1</i> reporter mice failed to detect. It is difficult to conclude whether TRPV1 is expressed in the basal ganglia.	(Cavanaugh et al., 2011; Cristino et al., 2006; Mezey et al., 2000)
Intrafascicular nucleus	Mouse, rat	Reporter gene expression, ISH	While previous experiments suggested the expression of TRPV1 in the region, the lack of positive/negative controls in the study design make it difficult to conclude whether TRPV1 is expressed in the intrafascicular nucleus.	(Cavanaugh et al., 2011)
Periaqueductal grey	Mouse, rat	IHC, reporter gene expression, pharmacological approaches	Given considerable supporting evidence, it is likely that TRPV1 is expressed in the periaqueductal grey.	(Cavanaugh et al., 2011; Cristino et al., 2006; Palazzo et al., 2002)

Region	Species	Technique	Critical evaluation	References
Supramammillary nucleus	Mouse, rat	Reporter gene expression, ISH	While previous experiments suggested the expression of TRPV1 in the region, the lack of positive/negative controls in the study design make it difficult to conclude whether TRPV1 is expressed in the supramammillary nucleus.	(Cavanaugh et al., 2011)

Table 1. Potential *Trpv1*-expressing brain structures in rodents – literature review.

1.4 TRPV1 as a potential drug target

Since TRPV1 has been well-established for its role in mediating pain, especially in inflammatory and chronic pain conditions, blocking TRPV1 activity has been proposed as a promising analgesic therapy. Two main approaches have been being investigated.

The first method involves the activation and following desensitization of TRPV1 channel (Brito et al., 2014). It should be noted that capsaicin, a prominent TRPV1 activator, has been traditionally used in relieving pain (Basith et al., 2016; Fattori et al., 2016). Recent studies have suggested the application of capsaicin in treating pain caused by arthritis and skin conditions, muscle damage, and peripheral neuropathy (Anand & Bley, 2011; Baranidharan et al., 2013; Bonezzi et al., 2020; Brederson et al., 2013; Kolasinski et al., 2020). The analgesic effect of capsaicin can be explained by prolonged activation of TRPV1 by capsaicin that would induce a calcium-dependent desensitization of TRPV1 and thus impair its ability to respond to capsaicin as well as other noxious stimuli (Joseph et al., 2013; Koplas et al., 1997; S. Wang et al., 2017). However, capsaicin's poor water solubility limits its bioavailability and efficacy *in vivo*. Moreover, painful burning sensations and the adverse effects on body temperature regulation have further decreased the application of capsaicin as a widely used therapeutic treatment option (Basith et al., 2016; Fattori et al., 2016; Gavva et al., 2008; Lu et al., 2020).

Further research focused on the development of other TRPV1 agonists that produce fewer, less harmful side effects than capsaicin, such as cis-capsaicin (cis-8-methyl-N-vanillyl-6-nonenamide) (Bourne et al., 1999; Sałat et al., 2014; Saper et al., 2002), trans-capsaicin (CNTX-4975) (Iftinca et al., 2020; Stevens et al., 2019), and CA-008 (Iftinca et al., 2020). Most of them are still undergoing clinical trials and would require further time and investigation for practical usage. One promising drug candidate is resiniferatoxin (RTX), a plant-derived ultrapotent agonist of TRPV1. RTX has been praised for the selective defunctionalization of TRPV1 and has proven itself as a potential treatment for highly localized pain (Brown, 2016; Pecze et al., 2017). However, the requirement of applying RTX to the exact site where the pain is generated remains a difficult challenge.

The second method involves the usage of TRPV1 antagonists to block TRPV1 receptors (Brito et al., 2014). The first reported TRPV1 antagonist, capsazepine can block capsaicin-induced TRPV1 activation in neonatal rats (S. Bevan et al., 1992b). However, due its low metabolic stability and poor selectivity, capsazepine was not considered for clinical usage (Docherty et al., 1997; Liu & Simon, 1997; Vriens et al., 2009; Weil et al., 2005). Despite its drawback, the discovery of capsazepine promoted development of new, more potent, and more selective TRPV1 antagonists. TRPV1 antagonists can be broadly classified into competitive and non-competitive antagonists. Competitive TRPV1 antagonists bound to the agonist binding site and locked TRPV1 in a closed, nonconductive state (Brito et al., 2014). Non-competitive TRPV1 antagonists interact with allosteric sites of TRPV1 and prevent channel opening such as ruthenium red and methocitramine (Brito et al., 2014). Many TRPV1 antagonists showed promising pain relieving effects in preclinical models such as BCTC, A-995662 and GRC-6211 (Iftinca et al., 2020)

One particular concern with using TRPV1 antagonists is that TRPV1 is also involved in regulating body temperature. Several TRPV1 antagonists have been found to induce hyperthermia in both rodents and humans (Garami et al., 2020; Gavva et al., 2008; Gavva, Bannon, Hovland, et al., 2007; Gavva, Bannon, Surapaneni, et al., 2007b). Interestingly, TRPV1 antagonists also resulted in hypothermia in certain cases (Garami et al., 2020). The mechanism by which TRPV1 antagonism led to different effects on body temperatures remains a challenging problem for the development of TRPV1-targeted drugs.

It can be concluded that despite existing difficulties and questions to be resolved, TRPV1 shows great potential as a promising drug target for treatment of various pain conditions.

2. Supramammillary nucleus

2.1 Structure and location

The supramammillary nucleus (SuM) is a set of nuclei located above the mamillary bodies (Pan & McNaughton, 2004). SuM can be classified as part of the hypothalamus (Pan & McNaughton, 2004; Vertes, 2015).

SuM is divided anatomically into two distinct regions, the lateral SuM (SuML) and the medial SuM (SuMM) (Paxinos et al., 2001). SuMM contains mostly smaller cells, while SuML contains larger cells (Pan & McNaughton, 2004). The schematic presentation of the mouse SuM can be found at Figure 2.

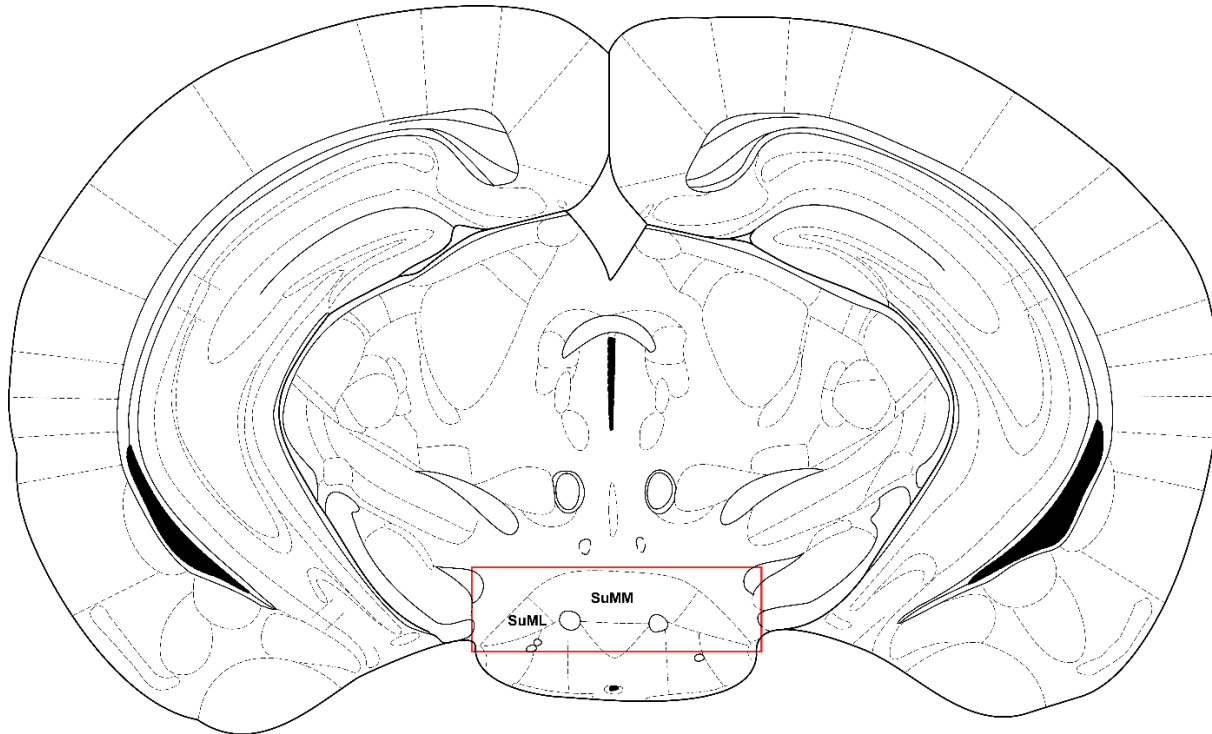


Figure 2. Mouse supramammillary nucleus. Bregma -2.8 mm. SuML: lateral supramammillary nucleus, SuMM: medial supramammillary nucleus (Paxinos et al., 2001).

2.2 Functions

Neurochemical anatomy and electrophysiological recordings have demonstrated the role of the SuM-hippocampus projections in the regulation of the theta and gamma oscillation in the dentate gyrus (Billwiller et al., 2020a; Kirk & McNaughton, 1991; Kocsis & Vertes, 1994; Kowalczyk et

al., 2021; Pedersen et al., 2017). SuM was also proposed to be involved in the local production of theta rhythmic oscillatory activity via the ascending brainstem hippocampal pathway (Kowalczyk et al., 2021).

Inactivation of SuM neurons in rodents was proven to impair the memory consolidation and retrieval but not the acquisition of spatial memory by behavioral tests, such as the passive avoidance test and the Morris water maze test (Aranda et al., 2006, 2008; Gutiérrez-Guzmán et al., 2012; Shahidi, Motamedi, & Naghdi, 2004; Shahidi, Motamedi, Bakeshloo, et al., 2004). Glutamate release from the mouse SuM was specifically suggested for its role in spatial memory retrieval in the novel place recognition test (Li et al., 2020). Recent studies also indicated the possible involvement of the SuM in social memory encoding (Robert et al., 2021).

Besides, it has been proposed that the SuM plays a certain role in rapid eye movement (REM) sleep, possibly via contributions to REM theta activity (Billwiller et al., 2020b; Pedersen et al., 2017; Renouard et al., 2015). Electrophysiological recordings from mouse brains further suggested the function of the SuM in wake-sleep regulation (Pedersen et al., 2017).

Activation of SuM glutamatergic neurons was shown to reinforce and energize behavioral interaction with the environment, possibly via the SuM-septal nucleus pathway (Kesner et al., 2021). Similarly, inhibition of SuM neurons disrupted sucrose-seeking behaviors in rats (Kesner et al., 2021). Injections of excitatory α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), γ -Aminobutyric acid type A (GABA_A) antagonist picrotoxin, and nicotine into the SuM induced rats to level-press for the selective self-administration of these compounds (Ikemoto, 2005; Ikemoto et al., 2004, 2006; Shin & Ikemoto, 2010). Besides, lesions in the rat SuM produced anxiolytic responses in the elevated T-maze test (Aranda et al., 2006). These evidence suggested the involvement of the SuM in regulating motivation and emotional behaviors.

2.3 Projections

Immunohistochemical staining, anterograde and retrograde tracing have demonstrated widespread projections from/to the SuM. SuM sends ascending projections to the hippocampus, entorhinal cortex, cingulate cortex, septal nucleus, and the amygdala (Carre & Harley, 1991; Haglund et al., 1984; Hashimotodani et al., 2018; Kesner et al., 2021; Kiss et al., 2000; Leranth & Kiss, 1996; Pan & McNaughton, 2004). Descending efferents from the SuM are mainly to the periaqueductal grey, median raphe, dorsal raphe, and the locus coeruleus (Pan & McNaughton, 2004).

In general, projections from the SuM follow a topographic pattern. SuMM projects primarily to medial structures, while cells from SuML projects to more lateral structures in the brain (Pan & McNaughton, 2004).

2.4 TRPV1 expression in the SuM: previous data

Trpv1 expression was detected in the SuM of *Trpv1* reporter mice (Figure 3) (Cavanaugh et al., 2011). Radioactive ISH also showed the expression of *Trpv1* mRNA in the mouse SuM (Cavanaugh et al., 2011). However, since the study failed to include any positive or negative control in the experimental design, we could question whether detected *Trpv1* mRNA expression was a valid finding in the mouse SuM. Besides, as it has been recognized that SuM is an important brain structure with numerous functions and widespread projections, it is possible that TRPV1 plays a significant role in several physiological processes in the SuM. However, very little information about the expression of TRPV1 in this brain region is available, including the type of neuron that potentially expresses TRPV1. Therefore, we aimed to further investigate *Trpv1* mRNA expression in the SuM.

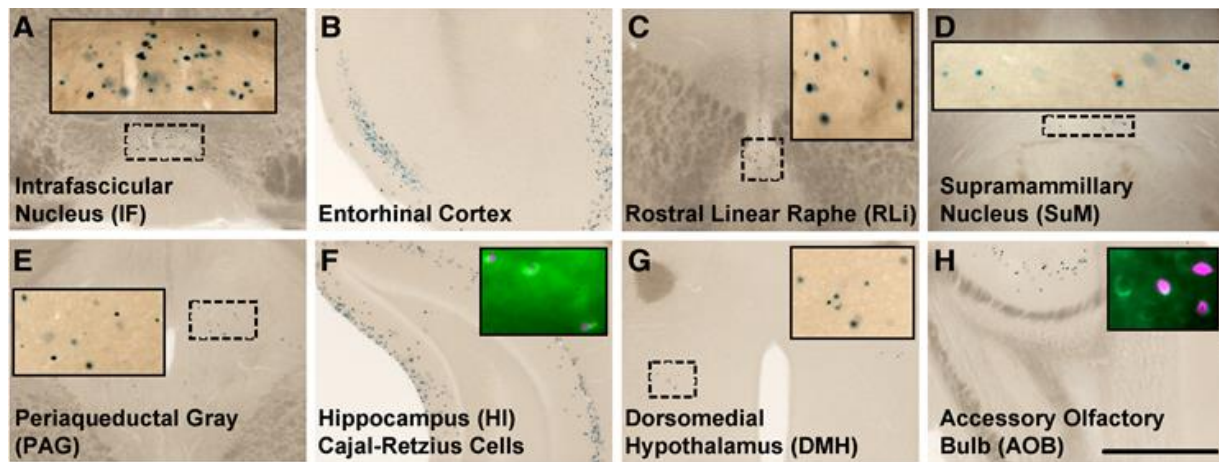


Figure 3. *lacZ* staining in coronal sections of *TRPV1^{PLAP-nlacZ}* mice. PLAP and *lacZ* are expressed under the control of the *Trpv1* promoter without disrupting the expression of *Trpv1*. *Trpv1* expression in the mouse supramammillary nucleus (SuM) was detected as blue dots in the image (D) (Cavanaugh et al., 2011).

3. Research goal

In this study, we plan to (i) examine the expression pattern of *Trpv1* in the mouse brain using RNAscope *in situ* hybridization technology (F. Wang et al., 2012).

From the singleplex RNAscope fluorescent results, we found that *Trpv1* is selectively expressed in the mouse SuM. We then further aim to (ii) characterize *Trpv1*-expressing cells in the mouse SuM by co-localizing *Trpv1* mRNA signals with SuM-specific neuronal markers, namely *Vglut2* (Vesicular glutamate transporter 2) mRNA (marker for glutamatergic neurons) and tyrosine hydroxylase IHC (marker for dopaminergic neurons).

Methodology

1. Sample preparation

All mice were bred and raised in the Animal House of the Department of Pharmacology and Pharmacotherapy of the University of Pécs following the regulations of the Animal Welfare Committee at the University of Pécs, the National Scientific Ethical Committee on Animal Experimentation in Hungary (permission no: BA02/2000/33/2018), and in agreement with the directive of the 1986 European Communities Council, as well as with the 1998 Law of XXCIII on Animal Care and Use in Hungary. 3–4-month-old male C57Bl/6 mice (N = 3) were deeply anesthetized with an overdose of urethan (2.4 g/kg) and transcardially perfused with ice-cold 0.1 M PBS (pH: 7.4), followed by 4% paraformaldehyde (PFA) solution in Millonig buffer (pH 7.4). Brains were dissected, postfixed for 24 hours at room temperature, rinsed in 0.1 M PBS, and stored at 4 °C. Brains were embedded in 4% freshly prepared agarose blocks and coronally sectioned (by 30 µm) using a Leica VT1000S vibratome (Leica Biosystems, Wetzlar, Germany). Sections were collected into RNase-free tubes and stored in 1x PBS-0.1% Na-azide at 4 °C.

Several brain regions within the mouse CNS (olfactory bulb, prelimbic cortex, septum, hypothalamus, hippocampus, piriform cortex, amygdala, primary somatosensory cortex, cerebellum, supramammillary nucleus) were sectioned and selected for singleplex RNAscope according to (Paxinos et al., 2001). Two representative sections from the mouse supramammillary nucleus (SuM, from Bregma -2.50 to -3.50 mm according to (Paxinos et al., 2001)) per animal were selected for RNAscope *in situ* hybridization combined with immunohistochemistry studies.

2. RNAscope *in situ* hybridization (ISH)

Singleplex RNAscope ISH was performed using RNAscope Multiplex Fluorescent Reagent Kit v2 (Advanced Cell Diagnostics (ACD), Newark, CA, USA) according to the manufacturer's protocol (N=3) with minor modification (Nemes et al., 2021). After tissue pretreatment, sections were

hybridized with probes specific to mouse *Trpv1* (ACD, Cat. No.: 313331-C2). Signal amplification and channel development were conducted sequentially. Cyanine 3 (Cy3) (1:750 diluted in TSA buffer) were used as fluorophores for *Trpv1* mRNA detection. Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI, ACD) and mounted with Prolong Glass antifade mountant (Thermo Fisher Scientific) for confocal imaging. Slides were stored at -20 °C.

Multiplex RNAscope ISH on mouse SuM sections was performed using the same procedure and reagent kit for singleplex RNAscope, with some minor differences. Pre-treated sections were instead hybridized with probes designed to mouse *Trpv1* (ACD, Cat. No.: 313331-C2) and *Vglut2* (ACD, Cat. No.: 319171-C3) mRNA. Cy3 (1:750 diluted in TSA buffer) and Cyanine 5 (Cy5) (1:1500 diluted in TSA buffer) were selected as fluorophores for *Trpv1* and *Vglut2* mRNA signals, respectively. RNAscope 3-plex mouse positive control probes (ACD, Cat. Nr.: 320881, probes specific to RNA polymerase II subunit A (*Polr2a*), peptidyl-prolyl cis-trans isomerase B (*Ppib*), ubiquitin C (*Ubc*)) and negative controls (ACD, Cat. Nr.: 320871, probe designed to bacterial, 4-hydroxy-tetrahydrodipicolinate reductase, *dapB*) were used in parallel to ensure interpretable and standardized results (N = 1).

3. Immunohistochemistry (IHC)

Tyrosine hydroxylase-specific immunohistochemistry was performed in coupled with multiplex RNAscope ISH according to the manufacturer's protocol (N=3) (TM323100, Tech Note: Dual ISH IHC manual Multiplex Fl v2, Advanced Cell Diagnostics). Sections were washed in 1% 0.1 M PBS and incubated with rabbit anti-tyrosine hydroxylase primary antibody (1:2000 diluted in 2% normal goat serum (NGS), Cat. Nr.: Ab112, Abcam, Cambridge, UK) overnight at room temperature. Sections were washed 3x15 min then incubated with Alexa 488-conjugated goat anti-rabbit secondary antibody (1:1000 pre-diluted in 2% NGS, A-11008, Thermo Fisher Scientific) for 3 hours at room temperature. For IHC, sections were counterstained with DAPI (ACD) and mounted with Prolong Glass antifade mountant (P36980, Thermo Fisher Scientific). Slides were stored at -20 °C for confocal imaging.

4. Confocal imaging

Fluorescent images (z-stacks with 1 µm intervals) of SuM according to (Paxinos et al., 2001) were acquired using a Zeiss LSM 710 confocal laser scanning microscope (Carl Zeiss AG, Oberkochen, Germany). Virtual colors were selected to depict fluorescent signals: green for Alexa 488 (tyrosine

hydroxylase IHC and *Polr2a* mRNA), red for Cy3 (*Trpv1* and *Ppib* mRNA), white for Cy5 (*Vlgut2* and *Ubc* mRNA), and blue for DAPI.

5. Image analysis

Brightness/contrast adjustment and z-projection (15 stacks/image, 1 μ m-intervals) with maximum intensity of separate channels were processed using (Fiji, 1.53c, NIH, USA).

Results

1. *Trpv1* was selectively expressed in the mouse supramammillary nucleus (SuM)

Significant *Trpv1* mRNA expression was specifically detected in the mouse SuM via singleplex fluorescent RNAscope (N = 3) (Figure 4).

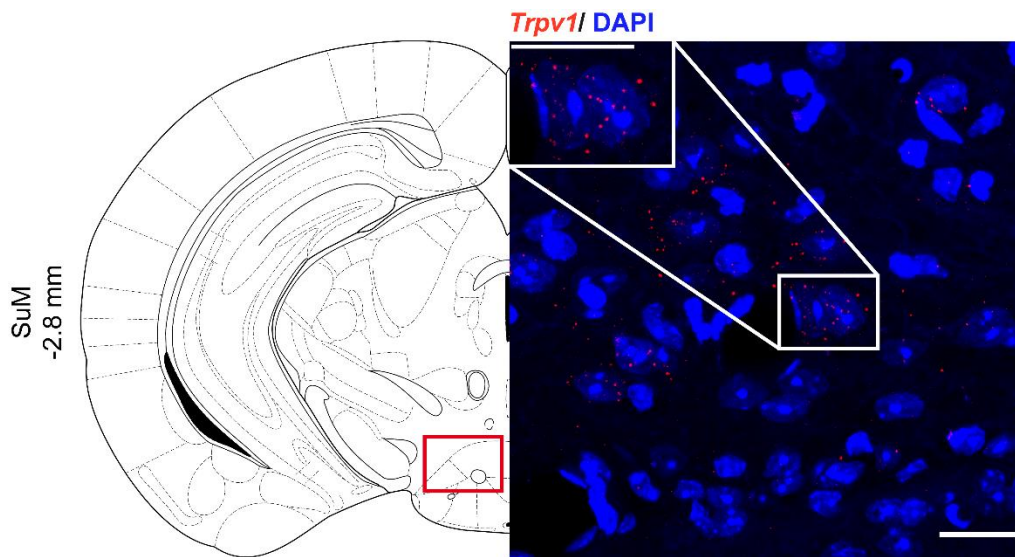


Figure 4. Representative confocal image of *Trpv1* mRNA expression in the mouse supramammillary nucleus (SuM, Bregma -2.8 mm, (Paxinos et al., 2001)). Sections were counterstained with DAPI (N=3). Scale bar 20 μ m, inset scale bar 20 μ m.

In contrast, brain regions with no *Trpv1* mRNA expression, such as olfactory bulb, prelimbic cortex, septum, hypothalamus, hippocampus, piriform cortex, amygdala, primary somatosensory cortex, cerebellum were also observed. Figure 5 illustrates negative *Trpv1*-specific singleplex RNAscope ISH results in the hippocampus, cerebellum, olfactory bulb, and the piriform cortex (other regions: images not shown).

2. *Trpv1* mRNA co-localizes with *Vglut2* mRNA but not with tyrosine hydroxylase immunopositivity in the mouse SuM

Next, using multiplex fluorescent RNAscope, strong co-localization between *Trpv1* and *Vglut2* mRNA was detected in the mouse SuM (N = 2) (Figure 6A). RNAscope and IHC multiplex fluorescent data found no co-localization between *Trpv1* mRNA and tyrosine hydroxylase immunopositivity (N = 2) (Figure 6B). Since we depicted confocal images of sections at 63x objective, we could only scan a small region within the mouse SuM, therefore we were unable to provide images where both the *Vglut2*-positive *Trpv1*-expressing glutamatergic neurons and the tyrosine hydroxylase-immunoreactive dopaminergic neurons were visible.

RNAscope 3-plex mouse positive control probes visualized moderate to strong signals of mouse housekeeping genes, *Polr2a* (RNA polymerase II subunit A), *Ppib* (peptidyl-prolyl cis-trans isomerase B), *Ubc* (ubiquitin C) (N=1) (Figure 6C). Negative controls for RNAscope (designed to bacterial *dapB* (4-hydroxy-tetrahydrodipicolinate reductase) gene) gave no detectable fluorescent signal (N=1) (Figure 6D).

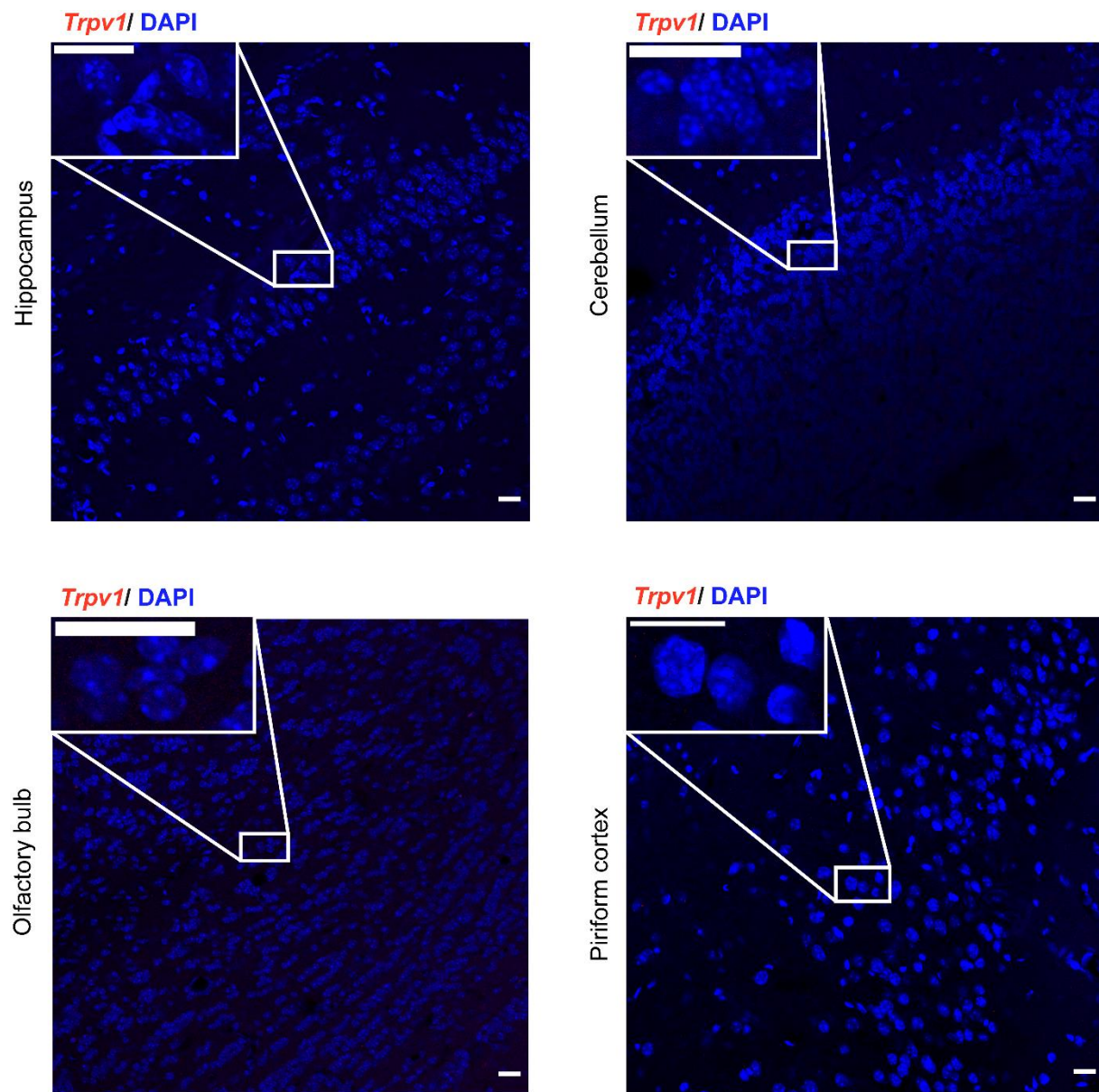


Figure 5. Negative *Trpv1*-specific RNAscope ISH results in mouse brains. No *Trpv1* mRNA expression was detected in the mouse hippocampus, cerebellum, olfactory bulb, piriform cortex. Scale bar 20 μm , inset scale bar 20 μm .

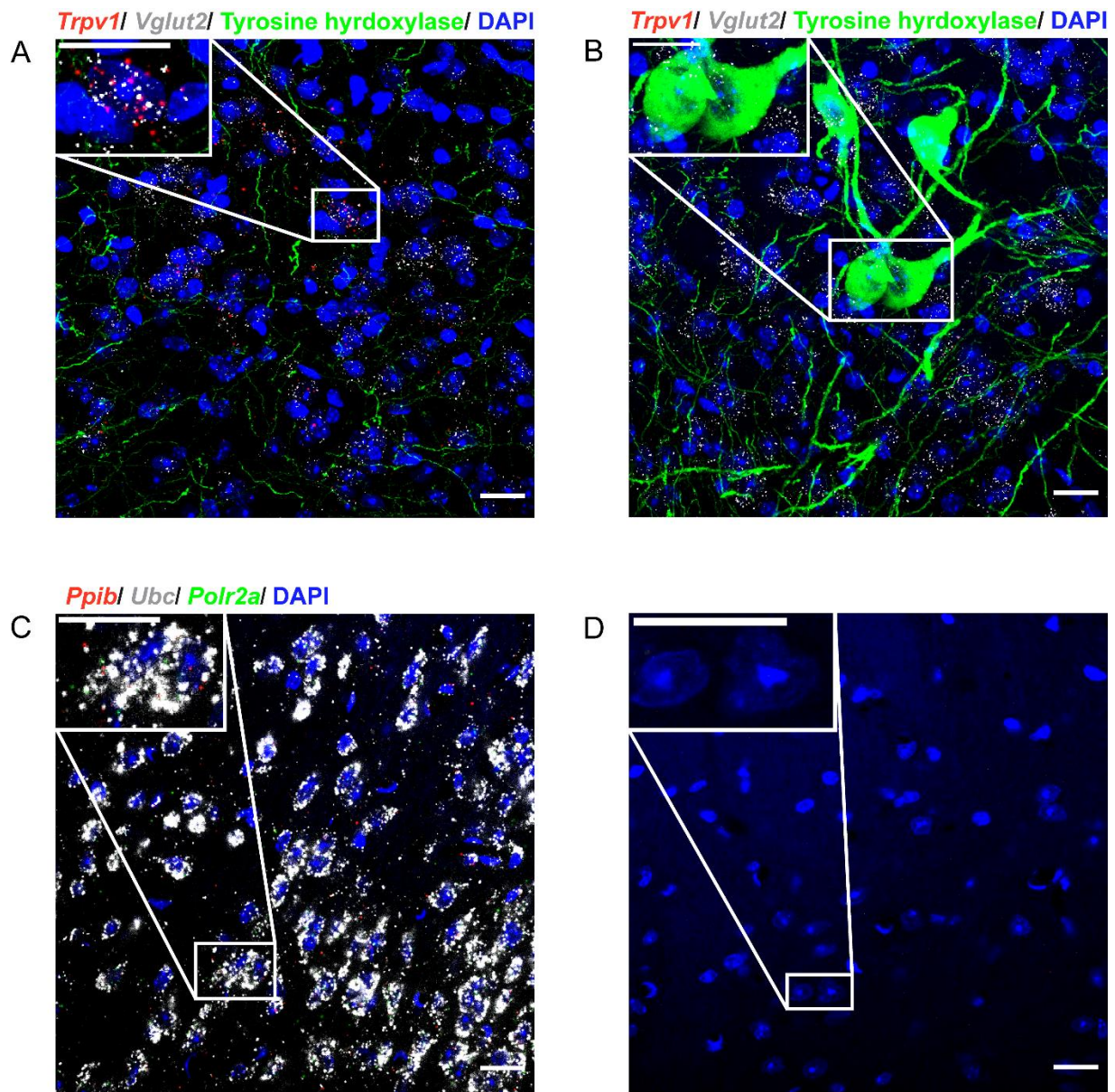


Figure 6. Representative RNAscope and IHC multiplex fluorescent staining in the mouse supramammillary nucleus. *Trpv1* mRNA co-localized with *Vglut2* mRNA (N=2) (A). There was no overlap between *Trpv1* mRNA and tyrosine hydroxylase immunoreactivity (N=2) (B). RNAscope 3-plex mouse positive control probes visualized moderate to strong signals of mouse housekeeping genes (N=1) (C). Negative controls for RNAscope gave no detectable fluorescent signal (N=1) (D). Nuclei were stained with DAPI. Scale bar 20 μ m, inset scale bar 20 μ m.

Discussion

In summary, we confirmed that *Trpv1* mRNA was selectively expressed in mouse SuM using ultrasensitive, fluorescent RNAscope *in situ* hybridization technology. Our results confirmed the

previously published *lacZ* reporter gene expression and radioactive ISH data in regard to the *Trpv1* expression in mouse SuM (Cavanaugh et al., 2011). Furthermore, as a novelty we showed that *Trpv1* mRNA expression in SuM was restricted to *Vglut2*-positive glutamatergic neurons, however, it was not co-localized with tyrosine hydroxylase-immunoreactive dopaminergic neurons. Fluorescent signals of mouse housekeeping genes (low copy *Polr2a*, medium copy *Ppib* and high copy *Ubc*) ensured that both the experimental design and findings were valid and reproducible. Results of negative controls for RNAscope assured that detected signals were not due to background staining artefacts or autofluorescence.

While *Trpv1* has been proposed to be broadly expressed within the CNS (for reference see Introduction 1.3), our results instead suggested a restricted expression of *Trpv1* within the mouse CNS, localized to the supramammillary nucleus specifically. Since our research group is the first group to utilize RNAscope ISH to examine *Trpv1* expression in the mouse CNS, it is possible that the usage of different technologies leads to inconsistent, potentially contradictory *Trpv1* expression data within the CNS. It can be explained that *Trpv1* is potentially expressed at different intensities across the CNS. Therefore, it is necessary to establish a standardized protocol to detect *Trpv1* expression in the CNS.

Trpv1 has been found to be expressed in the mouse SuM (Cavanaugh et al., 2011). Our data confirm the expression of *Trpv1* in this brain structure. In comparison to indirect detection via *Trpv1*-specific bacterial *lacZ* staining or traditional ISH with low sensitivity (Cavanaugh et al., 2011; Kobayashi et al., 2005), RNAscope, with its unique Z probe pair design and signal amplification cascade strategy (F. Wang et al., 2012), provided a highly specific, single molecule-sensitive detection of *Trpv1* mRNA signals. Besides, by visualizing each RNA molecule as a single dot, significant co-localization between *Trpv1* mRNA and *Vglut2* mRNA (glutamatergic marker) could be clearly detected and observed. This helped us to determine the neurochemical character of *Trpv1*-expressing neurons in the mouse SuM. However, since glutamatergic neurons in the mouse SuM differ in their functions and connections within the CNS (Billwiller et al., 2020a; Kesner et al., 2021; Pedersen et al., 2017), further experiments are required to characterize the exact neuronal subtypes that express *Trpv1* in the mouse SuM.

In addition, we provided a good example where multiplex RNAscope was an appropriate method to investigate the expression pattern of our transcript of interest (*Trpv1*) along with neuronal marker (*Vglut2*) similarly to previous studies (Kecskés et al., 2020).).

As a limitation, we could only perform expression analysis of *Trpv1* at the mRNA level instead of at the protein level. The expression pattern of *Trpv1* mRNA may not fully represent the distribution pattern of TRPV1 protein due to post-transcriptional modifications. Besides, RNAscope cannot reveal TRPV1 distribution within the *Vglut2*-positive neurons, we have no information whether TRPV1 as a functioning receptor is localized somatically in the SuM or at the axon terminals in one of the efferent projections (e.g. dentate gyrus, media preoptic area). This information would also be crucial for further experiments, such as Ca^{2+} imaging or in acute slice electrophysiology. Therefore, a commercially available, reliable anti-TRPV1 antibody would be crucial. However, the validity of previous studies using anti-TRPV1 antibodies have been challenged by contradicting findings (for reference see Table 1). For this reason, we were unable to perform TRPV1-specific IHC at the time of the experiments.

Conclusion and future perspectives

In summary, we concluded that glutamatergic neurons specifically express *Trpv1* in the mouse SuM. Glutamatergic neurons and their markers (e.g., *Vglut2* mRNA, VGLUT2 IHC) can act as indicators for the activity and functions of TRPV1 in the mouse SuM. Since TRPV1 itself is a Ca^{2+} channel (Stuart Bevan et al., 2014), we aim to investigate TRPV1 function in the mouse SuM using Ca^{2+} imaging and acute slice electrophysiology performed on SuM *Vglut2*-glutamatergic neurons or on efferent projections (see above in the Discussion section) Moreover, since the mouse SuM has been proposed to participate in several brain processes (e.g. memory, learning, wakefulness, for reference see Introduction 2.2), the combination of the corresponding behavioral tests (e.g., Morris water maze, Barnes maze) and *in vivo* techniques (e.g. Ca^{2+} imaging, administration of TRPV1 agonists/antagonists) in wild type compared to *Trpv1*^{-/-} mice would be necessary and appropriate methods to examine the physiological functions of TRPV1 in the mouse SuM.

It should be noted that many TRPV1 agonists and antagonists have been developed for the treatment of chronic pain, inflammation, and various diseases (for reference see Introduction 1.4). Since the SuM has been recently suggested for its involvement in anxiety-like behaviors (López-Ferreras et al., 2020), further studies into the role of TRPV1 in this region open the door for the development of new TRPV1-targeted drug candidates targeting the SuM for the treatment of mental illnesses. Besides, thorough understanding of TRPV1 expression in the CNS itself is particularly important to predict and explain the potential side effects of developing TRPV1-targeted drug candidates (Brito et al., 2014; Iftinca et al., 2020; Koivisto et al., 2021).

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Abbreviations

TRPV1	Transient receptor potential vanilloid 1 (protein)
<i>Trpv1</i>	Transient receptor potential vanilloid 1 encoding gene in rodents
TRP	Transient receptor potential
<i>Trpv1</i> ^{-/-} mice	<i>Trpv1</i> -deficient mice
PNS	Peripheral nervous system
CNS	Central nervous system
ISH	<i>In situ</i> hybridization
IHC	Immunohistochemistry
RT-PCR	Reverse transcription PCR
SuM	Supramammillary nucleus
SuML	Lateral supramammillary nucleus
SuMM	Medial supramammillary nucleus
<i>Vglut2</i>	Vesicular glutamate transporter 2 encoding gene in rodents
VGLUT2	Vesicular glutamate transporter 2
DAPI	4',6-diamidino-2-phenylindole
<i>Polr2a</i>	RNA polymerase II subunit A
<i>Ppib</i>	Peptidyl-prolyl cis-trans isomerase B
<i>Ubc</i>	Ubiquitin C
<i>dapB</i>	4-hydroxy-tetrahydrodipicolinate reductase

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