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Metabotropic glutamate receptor 6 signaling enhances TRPM1 calcium channel function and increases melanin content in human melanocytes

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SUMMARY

Mutations in TRPM1, a calcium channel expressed in retinal bipolar cells and epidermal melanocytes, cause complete congenital stationary night blindness with no discernible skin phenotype. In the retina, TRPM1 activity is negatively coupled to metabotropic glutamate receptor 6 (mGluR6) signaling through $G\alpha_o$ and TRPM1 mutations result in the loss of responsiveness of TRPM1 to mGluR6 signaling. Here, we show that human melanocytes express mGluR6 and treatment of melanocytes with L-AP4, a type III mGluR-selective agonist, enhances Ca^{2+} uptake. Knockdown of TRPM1 or mGluR6 by shRNA abolished L-AP4-induced Ca^{2+} influx and TRPM1 currents showing that TRPM1 activity in melanocytes is positively coupled to mGluR6 signaling. $G\alpha_o$ protein is absent in melanocytes. However, forced expression of $G\alpha_o$ restored negative coupling of TRPM1 to mGluR6 signaling, but treatment with and pertussis toxin, an inhibitor of G_i/G_o proteins, did not affect basal or mGluR6-induced Ca^{2+} uptake. Additionally, chronic stimulation of mGluR6 altered melanocyte morphology and increased melanin content. These data suggest differences in coupling of TRPM1 function to mGluR6 signaling explain different cellular responses to glutamate in the retina and the skin.

Keywords

TRPM1; cCSNB; Melanocytes; mGluR6; G proteins; GTPγS; GNAO1

INTRODUCTION

Transient receptor potential, melastatin 1 (*TRPM1*), a member of the TRPM subfamily of TRP proteins, is a calcium channel expressed in melanocytes and the retinal ON bipolar cells (Devi et al., 2009, Oancea et al., 2009, Kim et al., 2008, Shen et al., 2009). In epidermal melanocytes, TRPM1 expression is associated with proliferation and melanin

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pigmentation (Devi et al., 2009, Oancea et al., 2009). TRPM1 plays a critical role in the synaptic transmission from photoreceptors to ON bipolar cells (Morgans et al., 2009, Schmidt, 2009). Mutations in human *TRPM1* are associated with complete congenital stationary night blindness (cCSNB) and the photoresponse of ON bipolar cell to light is completely abolished in the *Trpm1*-null mouse (van Genderen et al., 2009, Audo et al., 2009, Li et al., 2009, Nakamura et al., 2010, Peachey et al., 2012). Intriguingly, patients with cCSNB do not exhibit any discernible skin pigment phenotype or photosensitivity (Li et al., 2009); and the *Trpm1*-null mouse has normal coat color phenotype (Koike et al., 2010a). However, several breeds of horses with Leopard complex spotting mutation (LP), which results in reduced TRPM1 mRNA expression in skin and retina, exhibit both night blindness and spotted coat pattern (white spotting centered around hips with or without spots of pigment) (Bellone et al., 2008, Bellone et al., 2010). The basis for these phenotypic differences in the effects of TRPM1 mutations is not known.

In retinal ON bipolar cells, TRPM1 is a component of metabotropic glutamate receptor 6 (mGluR6) signaling pathway and is shown to be negatively regulated by the G protein $G\alpha_o$ (Koike et al., 2010a, Koike et al., 2010b). Recently, however, it was reported that $G\beta\gamma$ dimer, but not $G\alpha_o$, is required for the G protein-mediated inhibition of the TRPM1(Shen et al., 2012). Although glutamate is known to affect melanin pigmentation (Frati et al., 2000), the role of mGluR6 and G-proteins in regulation of TRPM1 function in melanocytes is not clear. To understand whether differences in coupling of TRPM1 to mGluR6 signaling could explain the lack of effect of TRPM1 mutations on melanocytes , we investigated mGluR6 signaling and its role in regulation of TRPM1 channel activity in human melanocytes. Here, we show that human melanocytes express mGluR6 but not its downstream mediator $G\alpha_o$; and in contrast to retinal cells, stimulation of mGluR6 in melanocytes enhances TRPM1 channel activity and calcium influx and transient expression of $G\alpha_o$ in melanocytes was sufficient to restore negative coupling of TRPM1 to mGluR6 stimulation. Moreover, chronic stimulation of mGluR6 caused an increase in melanin content in human melanocytes. Our data suggest that difference in $G\alpha_o$ expression and coupling of TRPM1 to mGluR6 signaling seem to produce different phenotypic effects of mGluR6 stimulation.

RESULTS

Expression of mGluR6 and its effect on TRPM1 function in melanocytes

Quantitative RT-PCR analyses showed mGluR6 as the most abundant and the predominant group III mGluR mRNA expressed in human melanocytes. mGluR6 protein was readily detectable in melanocytes cultured under conditions that favor either proliferation or differentiation (Supporting Information, Figs. S1A & B). To test whether mGluR6 activation affects Ca^{2+} flux in melanocytes, we measured intracellular Ca^{2+} transients after stimulation with mGluR6 agonist L-AP4, a glutamate analogue and selective agonist for group III metabotropic glutamate receptors (Shiells et al., 1981, Slaughter and Miller, 1981). We loaded melanocytes with the ratiometric indicator dye Fura-2, added L-AP4 to the imaging bath solution for 30s and measured Ca^{2+} uptake following depletion of intracellular Ca^{2+} store by thapsigargin. As shown in Fig. 1A, brief stimulation of melanocytes with 50μM L-AP4 caused a significant increase in Ca^{2+} uptake, which was abolished by knockdown of mGluR6 by shRNA lentivirus (Fig. 1B). Knockdown of TRPM1 also attenuated or completely abolished L-AP4-induced increase in intracellular $[Ca^{2+}]$ (Fig. 1C) showing that TRPM1 is required for mGluR6 stimulated Ca^{2+} uptake by melanocytes. To test whether the L-AP4 induced elevation of intracellular $[Ca^{2+}]$ was due to influx through the TRPM1 channel, we measured Ca^{2+} uptake in the presence of $ZnCl_2$, an inhibitor of TRPM1 channel function (Lambert et al., 2010). Addition of $ZnCl_2$ to the extracellular medium caused a dose-dependent inhibition of constitutive as well as L-AP4-induced Ca^{2+} uptake by

melanocytes (Fig. 1D). These data show that in melanocytes mGluR activation increases Ca^{2+} influx through TRPM1.

Whole-cell patch clamping studies confirm a role for mGluR6 in TRPM1 function

We measured the effect of L-AP4 stimulation on TRPM1 channel currents in primary melanocytes using whole-cell patch clamp method. Detection of TRPM1 current is complicated by constitutive TRPM7 (Oancea et al., 2009). We included 8mM Mg^{2+} in intracellular recording solution to inhibit the endogenous TRPM7 currents and measured TRPM1 currents using step protocol (Fig. 2A). Melanocytes yielded an average peak outward current of 11.5 ± 2 pA/pF at 120 mV and in cells transduced with a TRPM1 shRNA lentivirus, the peak outward current was significantly reduced (−1 ± 0.4 pA/pF) (Fig. 2A), suggesting that the outward current observed in melanocytes is specific to the TRPM1 channel.

To confirm the role of mGluR6 receptor on TRPM1 ion channel current, we measured the inward TRPM1 currents by holding cells at 0 mV. Following baseline recording of current for 5 sec, the cells were perfused with L-AP4 (10 μM), which caused a rapid inward current in the control shRNA lentivirus transduced cells (average 2.4 pA/pF). Knockdown of TRPM1 completely abolished the L-AP4-induced inward current showing that glutamate receptor stimulated inward current requires TRPM1 channels (Fig. 2B). Similarly, knockdown of mGluR6 resulted in a significant reduction in the L-AP4 stimulation of TRPM1 channel activity whereas scrambled control shRNA did not affect the L-AP4 induced TRPM1 current. Consistent with the Ca^{2+} imaging data, patch clamp recordings show positive coupling of TRPM1 channel function with mGluR6 signaling.

Role of $G\alpha_o$ (GNAO) in mGluR6 signaling in melanocytes

In the retinal ON bipolar cells, negative regulation of TRPM1 channel activity by mGluR6 is mediated by the G protein $G\alpha_o$ (GNAO), a protein expressed abundantly in neuronal tissues, peripheral neurons and projections. In melanocytes, however, $G\alpha_{o1}$ protein was not detectable (Fig. 3A). We then asked whether forced expression of $G\alpha_o$ (by transient transfection) could restore negative coupling of TRPM1 Ca^{2+} channel function to mGluR6 signaling. We used co-expression of mCherry to identify transfected cells for ratiometric fluorescence imaging. Stimulation with L-AP4 caused significantly lower Ca^{2+} influx in melanocytes transfected with $G\alpha_{oA}$ or $G\alpha_{oB}$ compared to vector transfected cells (Fig. 3B) showing $G\alpha_o$ expression is sufficient for negative coupling of TRPM1 to mGluR6 signaling in melanocytes. Recently, $G\beta\gamma$ dimer has been reported to also play a role in the mGluR6-TRPM1 signaling. Therefore, we asked whether the absence of negative regulation of TRPM1 by mGluR6 in melanocytes is due to the absence of $G\beta\gamma$ proteins. Western blot analysis confirmed the expression of Gβ1 in four independent isolates of human epidermal melanocytes (Fig. S2B). In addition, expression of Gβ proteins Gβ2 and Gβ5 (GNB2 and GNB5) and Gγ (GNG2, GNG5 and GNG7) subunit proteins in melanocytes has been reported (http://www.proteinatlas.org/) (Yajima et al., 2012). Thus, melanocytes can use Gαo in a classic heterotrimer complex to close the channel. These data suggest that differences in $G\alpha_o$ expression explain cell type differences in coupling of TRPM1 channel to mGluR6 signaling.

Next, we asked whether any other endogenous Gα proteins belonging to family of the pertussis toxin (PTX)-sensitive G proteins are expressed in melanocytes and participate in coupling TRPM1 channel function to mGluR6 signaling cascade. Western blot analysis showed that melanocytes express $G\alpha_{i1}$ and $G\alpha_{i3}$, but not $G\alpha_{i2}$ (Fig. S2A). Forced expression of $G\alpha_{i2}$ or transducin (G-protein primarily involved in visual transduction by rhodopsin) resulted in marked inhibition of Ca^{2+} influx upon L-AP4 stimulation (Fig. S2C).

Although forced expression of PTX-sensitive proteins caused significant inhibition of Ca^{2+} influx in response to L-AP4 stimulation, treatment of melanocytes with PTX (and inhibition of endogenous G proteins), had no effect on either basal or L-AP4-stimulated Ca^{2+} influx (Fig. 3C).

We then asked whether GTP-binding protein(s) and GTP hydrolysis are required for L-AP4-induced increase in TRPM1 activity. We treated melanocytes with GTPγS, a non-hydrolysable GTP analog, and measured basal and L-AP4-induced Ca^{2+} influx. As shown Fig. 3D, incubation of cells with GTPγS alone caused a maximal Ca^{2+} influx nearly to the same magnitude as in L-AP4 treated cells suggesting that binding of GTP to GTP-binding protein(s) was sufficient to enhance Ca^{2+} uptake similar to stimulation of mGluR6. However, GTPγS had no effect on L-AP4 induced Ca^{2+} influx. These data suggest that although Ca^{2+} uptake by melanocytes is stimulated by GTP, its hydrolysis is not required for stimulation of TRPM1 channel function by mGluR6 signaling.

Effect of glutamate and L-AP4 on melanocyte functions

To investigate the effect of chronic stimulation of mGluR6, we cultured melanocytes in medium with no glutamate or varying concentrations of glutamate or L-AP4. After 6 days of culture in glutamate, melanocytes showed distinct morphological changes with shorter, wider and stubby dendrites compared to the long and slender dendrites in glutamate-free medium (Fig. 4A & S3A). Such changes in dendrite morphology were more prominent in cells cultured with L-AP4 and showed a dose-dependent change (with >70% decrease in dendrite length and 50% increase in dendrite width after 6-day exposure to 50 μM L-AP4) than cells cultured with glutamate (Fig. 4B and Fig. S3B). L-AP4 treatment also reduced the melanocyte cell number and increased intracellular melanin content (Figs. 4C & S3C). However, neither treatment resulted in any significant change in tyrosinase protein or tyrosinase activity (Fig. S3D & S3E and data not shown). These data suggest that chronic activation of mGluR6 promotes melanocyte differentiation and melanin production.

DISCUSSION

In previous studies, we and others showed that TRPM1 activity contributes significantly in Ca^{2+} uptake by melanocytes and TRPM1 expression is associated with melanocyte proliferation and melanin pigmentation *in vitro* (Devi et al., 2009, Oancea et al., 2009). A role for TRPM1 in pigmentation is also indicated by a relationship between the spotted coat color and reduced TRPM1 expression observed in the skin of several breeds of horses (Bellone et al., 2008, Bellone et al., 2010, Sandmeyer et al., 2007, Sandmeyer et al., 2012). However, the reasons for the lack of skin or pigmentation abnormalities in the limited number of cCSNB patients studied and lack of coat color phenotype in *Trpm1*$^{-/-}$ and Trpm mutant mice are not yet known (Audo et al., 2009, Koike et al., 2010b, Li et al., 2009, Nakamura et al., 2010, Peachey et al., 2012, van Genderen et al., 2009).

This study highlights the possible reasons for the differences between melanocytes and retinal ON bipolar cells in their responses to mGluR6 signaling. A functional regulatory cascade between mGluR6 and TRPM1 has been demonstrated in dark adaption of retinal ON bipolar cells via the activation of G protein cascade involving $G\alpha_o$ (Weng et al., 1997, Nawy, 1999, Dhingra et al., 2000, Dhingra et al., 2002). A recent report has, however, implicated Gβγ, rather than $G\alpha_o$, in the negative regulation of TRPM1 by G-protein (Shen et al., 2012). There are limited studies on expression of mGluR and Gα and their role in melanocyte biology. (Ohtani et al., 2008, Marin et al., 2005, Shin et al., 2008, Frati et al., 2000). In this study, we found that human melanocytes express mGluR6 but $G\alpha_o$ protein was not detectable in human melanocytes, even though $G\alpha_o$ mRNA was detectable. Expression of mGluR6 in melanocytes raised the possibility that TRPM1 in melanocytes is

also negatively regulated by the mGluR6 signaling similar to that seen in retinal bipolar cells, albeit through a non-$G\alpha_o$ mediator. We found that glutamate and the Type III mGluR agonist caused a significant increase in calcium uptake. Glutamate acts on both metabotropic and ionotropic receptors coupled to GTP binding proteins with a high affinity for group I mGluRs. A similar increase in calcium influx observed with L-AP4, a group III specific agonist (Shiells et al., 1981, Slaughter and Miller, 1981), and inhibition of its effect by genetic knockdown of mGluR6 or TRPM1, confirmed both the existence of mGluR6-TRPM1 circuit and a positive coupling of TRPM1 to mGluR6 signaling in melanocytes. Although L-AP4 is known to activate mGluR4 and mGluR6 at low concentrations (5-50μM), at higher concentrations it also activates mGluR7 and mGluR8 (Niswender and Conn, 2010, Schoepp et al., 1999, Pin and Duvoisin, 1995), we saw maximal response in melanocyte at 50 μM with no additional increase in calcium uptake at higher concentrations suggesting a saturable effect of this metabotropic glutamate receptor agonist.

Our finding that human melanocytes do not express $G\alpha_o$ protein and its forced expression is sufficient to restore the negative coupling of TRPM1 activity to mGluR6 activation supports the notion that differential coupling of TRPM1 and glutamate signaling is due to association of different Gα proteins with mGluR6 in retinal ON bipolar cells and epidermal melanocytes. In this context, although it is tempting to consider a role for Gβγ in regulation of TRPM1, it should be noted that in their studies Shen et al. also found a negative coupling of TRPM1 to mGluR6 signaling (Shen et al., 2012). At this time, the reasons for this discrepancy are not entirely clear, but could be related to methodological differences and/or the use of different mGluR6 agonists/antagonists and heterologous cell systems employed. However, Gβγ proteins are expressed in melanocytes (Fig. S2B). Indeed the expression of the three G-protein subunits in the heterotrimer is known to be coordinated and expression of one subunit affects the expression of the other two (Dhingra et al., 2012, Marrari et al., 2007). We also tested the involvement of other G proteins sensitive to pertussis toxin, which causes ADP-ribosylation in some of the G proteins (G_i, G_o and transducin) and making them inactive (Milligan, 1988) and found that PTX-sensitive G proteins are not involved mGluR6-TRPM1 signaling pathway in melanocytes. Although inhibitory actions mediated through a PTX-sensitive G proteins are well documented (Kleuss et al., 1991, Pin and Duvoisin, 1995), there are only limited reports of involvement of PTX-refractory G protein mediated excitatory actions (Nakajima et al., 1988). Therefore, it is possible that in melanocytes the stimulatory G protein (G_s) mediates this effect. We noted that GTPγS, which irreversibly activates G proteins in the absence of agonists (Breitwieser and Szabo, 1985, Holz et al., 1986), caused a significant increase in calcium uptake, presumably through TRPM1, without stimulation of mGluR6 suggesting that some GTP-binding protein(s) are involved in L-AP4 mediated increase in calcium uptake. Additional biochemical experiments are needed to identify such GTP-binding proteins.

The physiological activator(s) and inhibitor(s) of TRPM1 in melanocytes have not yet been identified. Zn^{2+} has been shown to cause a concentration dependent inhibition of TRPM1 channel functions (Lambert et al., 2010) and other TRPM channels (Yang et al., 2011). We found that Zn^{2+} caused a dose dependent decrease in basal Ca^{2+} uptake by melanocytes upon internal store depletion. In the presence of Zn^{2+}, L-AP4 failed to increase Ca^{2+} uptake, supporting a role for TRPM1 in L-AP4 stimulated increase in Ca^{2+} influx. These data provide additional support for positive coupling between mGluR6 and TRPM1 in melanocytes. Interestingly, TRPM1 conducts outwardly rectifying current in melanocytes similar to the current in retinal bipolar cells (Shen et al., 2009) and L-AP4 evoked TRPM1 current was significantly reduced by knockdown of mGluR6. This is in contrast to the reduced TRPM1 current by glutamate seen in CHO cells expressing TRPM1 and mGluR6 in presence of expressed $G\alpha_o$ (Koike et al., 2010b). The reasons for this discrepancy are not clear. But, differences due to overexpression in heterologous cells or absence of TRPM1

splice variants and/or other protein factors normally present in melanocytes could contribute to these differences. Our electrophysiological data clearly show that TRPM1 mediated current in melanocytes is regulated by the mGluR6 signaling pathway similar to retinal ON bipolar cells, but mGluR6 activation in melanocytes produces an opposite effect on TRPM1 current.

Physiological significance of the effects of glutamate, specifically activation of mGluR6, in skin pigmentation is not well understood. We found that chronic stimulation of melanocytes with L-AP4 caused pronounced change in dendritic morphology. Dendritic morphology is intimately associated with melanocyte function, i. e., transport and export of melanosomes to neighboring keratinocytes. Glutamate toxicity and neuronal injury has been well studied and effect of glutamate on neuronal dendrites has been reported (Metzger et al., 1998). In contrast to our findings, Hoogduijn and group did not find a toxic effect of glutamate on melanocytes whereas Frati el al reported more than 70% cell death to 1mM glutamate (Frati et al., 2000, Hoogduijn et al., 2006). Interestingly, we found that treatment with L-AP4 also resulted in an increase in cellular melanin content, presumably due to accumulation of melanized melanosomes. This raises the possibility that glutamate receptor activation in epidermal melanocytes inhibits export of melanosomes to keratinocytes in humans while the opposite may be true in the horse. Additional studies are needed to understand the detailed mechanisms of cutaneous glutamate receptor signaling network and its role in the regulation of skin pigmentation and pigmentary disorders. For example, it is possible that physiological conditions or environmental influences that alter concentration of mGluR6 agonists in the skin might uncover pigmentary defects in patients with cCSNB,

In summary, our study shows that glutamate signaling pathway, a widely studied signaling mechanism in the neuronal system, is also involved in cutaneous melanocyte proliferation and differentiation. Therefore, it will be worthwhile to investigate whether agents that modulate the GPCR activity of mGluR6 or the ion-channel activity of TRPM1 or downstream targets of the mGluR6-TRPM1-Ca^{2+} signaling are useful to treat hypo- and hyperpigmentary disorders.

METHODS

Cell culture

Human melanocytes were cultured from neonatal foreskins as described previously (Devi et al., 2009). All human subjects research described in this study were approved by the Institutional Review Board of University of Wisconsin. All tissue culture media and supplements were purchased from Invitrogen (Carlsbad, CA). TPA, IBMX, CT and L-glutamate were from Sigma Aldrich (St. Louis, MO). Cells were photographed using 20X objective on Nikon inverted microscope. For measurement of dendrite length and width, cells (n ≥300) in 5 randomly chosen fields were used for each treatment group. Total melanin content was determined as previously described (Devi et al., 2009).

Antibodies

Polyclonal anti-mGluR6 (ab90866) antibody was from Abcam (Cambridge, MA). Mouse monoclonal (mAb) anti-$G\alpha_o$ (MAB3073) was from EMD Millipore (Billerica, MA); mAbs anti-$G\alpha_{i1,}$ (sc-13533), $G\alpha_{i2}$ (sc-13534) and rabbit polyclonal anti-$G\alpha_{i3}$ (sc-262) were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit anti-humanTRPM1 antibody was described previously (Devi et al., 2009).

Lentiviruses and transduction

Short hairpin (sh) RNA plasmid targeting mGluR6 (NM_000843) and a scrambled control shRNA plasmid were purchased from Open Biosystems (Huntsville, AL). Five shRNAs for mGluR6 (TRCN9028-TRCN9032, referred to here as sh–28 to sh-32) targeting nucleotides between 4221-4241 (sh-28), 1547 to 1567 (sh-29), 1250-1270 (sh-30), 2620-2640 (sh 31), 2346-2356 (sh-32) of mGlu6 RNA were tested for the efficiency and specificity of knockdown of mGluR6. Lentiviruses for mGluR6 and TRPM1 shRNA were generated as described previously (Devi et al., 2009). The efficiency of mGluR6 shRNAs was tested by transducing Jurkat cells that show abundant expression of mGluR6 and selected two shRNAs based on the efficiency of knockdown as assessed by western blotting.

Calcium imaging

Melanocytes plated on glass chamber slides were washed in buffer D (153mM NaCl, 5.4mM KCl, 1.8mM $CaCl_2$, 10mM N-2-hydroxy ethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 25mM glucose, 0.8mM $MgSO_4$, 0.9mM NaH_2PO_4 and 2.4mM $NaHCO_3$, pH 7.4), incubated in 2 μM Fura-2 (Invitrogen) in buffer D at 37^0C for 60 minutes, washed three times with buffer D without calcium and de-esterified for 30 minutes at 37^0C. Cells were imaged and ratio of fluorescence emission was monitored using Nikon Ti fluorescence microscope and NIS-Elements software. In a 20x field with well separated cells (n=8-10), baseline was established in buffer D without Ca^{2+} and change in F340/380 was monitored after addition of various reagents or Ca^{2+} for a total of 15 minutes with no cell exposed to light for longer than 20 min. Results were expressed as ratio after background fluorescence subtraction and normalization to baseline fluorescence.

Whole-cell patch clamping

Melanocytes grown on collagen coated cover slips for 72-96 h were used for whole cell patch clamp analysis using Axopatch 200B amplifier (Axon Instruments, Foster City, CA) with pCLAMP 10.2, in voltage clamp mode. For measuring current densities a step protocol was applied at holding potential of 0 mV in 10 mV steps from −120 mV to +120 mV for the duration of 250ms. The data were filtered at 5 kHz and digitized at 50 kHz. The current traces were corrected for linear capacitance and leak using −P/4 subtraction. Cells were perfused with normal physiological bath buffer containing (in mM) 140 Sodium gluconate, 4 NaCl, 2 $MgCl_2$, 10 HEPES, 10 Glucose, 2 $CaCl_2$, (pH-7.4, adjusted with NaOH). The patch pipettes were pulled from thin walled borosilicate glass capillaries (World Precision Instruments, Inc., Sarasota FL) and filled with an intracellular recording solution containing the following (in mM) 100 CsMES,10 Cs4BAPTA, 8 $MgCl_2$, 10 HEPES,1 $CaCl_2$, 0.3 Na-ATP (pH=7.2 was adjust with MES). All experiments were carried out at room temperature with pipette resistance of 1.5–2.5MΩ. To measure agonist stimulated specific *TRPM1* activity the cells were held at a holding potential of 0 mV and peak inward currents were measured in a continuous mode. Following a baseline current measurement, the cells were stimulated with group III metabotropic glutamate receptor agonist L-AP4 (10 μM) by perfusing the agonist into bath solution.

Statistical analysis

Nonparametric, one way ANOVA and Bonferroni's multiple comparison tests (Prism, GraphPad Software, La Jolla, CA) were used to compare the cell growth, melanin content, dendritic length and dendritic width. Fluorescence intensity data were analyzed by Student's *t*-test and are shown as mean ± SEM. Two-tailed *P* values <0.05 were considered statistically significant. For electrophysiological studies, the data were analyzed using Origin software (OriginLab Corporation, Northampton, MA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

L-AP4	L-2-amino-4-phosphonobutyrate
PTX	pertussis toxin
mGluR6	metabotropic glutamate receptor 6
cCSNB	complete congenital stationary night blindness
GTPγS	guanosine 5′-O (3-thio -triphosphate)

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SIGNIFICANCE

Mutations in *TRPM1*, a gene highly expressed in melanocytes and retinal bipolar cells, cause congenital night blindness but no overt skin pigmentary phenotype. The visual defect is due to the non-responsiveness of the mutant TRPM1 to glutamate signaling. In this study, we show that absence of the G protein $G\alpha_o$ in melanocytes results in differential coupling of TRPM1 function to glutamate receptor signaling. We show that mGluR6 signaling enhances TRPM1 activity in melanocytes and also increases melanin pigmentation. Our data provide a possible explanation for lack of pigmentary defects in TRPM1 mutants and suggest that selective expression of Gα subunit proteins can produce cell type-specific TRPM1 mutant phenotypes.

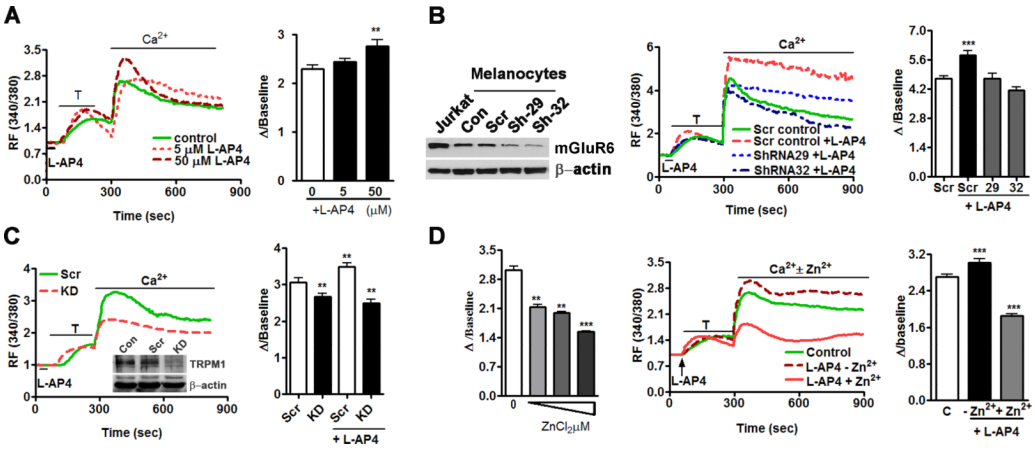

Fig. 1. mGluR6- and TRPM1-dependent effect of L-AP4, a group III mGluR-selective agonist, on calcium uptake by melanocytes

A) *Left:* Cytoplasmic calcium concentration in cultured neonatal human foreskin melanocytes loaded with Fura-2/AM was monitored and measured using ratiometric fluorescence imaging. Thapsigargin (T; 3μM) and calcium chloride (Ca^{2+}; 1.8mM) were added to bath solution for periods indicated by the span of the horizontal lines. *Right:* Mean relative fluorescence ratio calculated for 10-12 cells (up to 50 ROIs) is shown after subtraction of background fluorescence and normalization to baseline fluorescence ratio recorded prior to addition of thapsigargin. Maximal change in fluorescence ratio for all cells in each group is shown (**p<0.01). **B)** Knockdown of mGluR6 abolishes L-AP4-induced increase in calcium uptake. *Left:* Western blot analysis of efficacy of mGluR6 knockdown. Primary melanocytes were either mock transduced (Con) or transduced with scrambled (Scr) shRNA or mGluR6 shRNA lentiviruses (sh-29 and sh-32). Jurkat cells and β-actin served as positive control and protein loading control, respectively. *Middle:* mGluR6 knockdown abolished the L-AP4 induced increase in Ca^{2+}. Cells cultured on glass coverslip slides were transduced with control (scrambled) and mGluR6 shRNA lentiviruses, loaded with Fura-2 and changes in [Ca^{2+}] was monitored as described in **A**. *Right:* Maximal change in fluorescence ratio for all cells in each group is shown (*** *Scr vs Scr+L-AP4* p<0.001; Scr +L-AP4 vs sh29 p<0.05, Scr+L-AP4 vs sh32 p<0.001). **C)** TRPM1 is required for L-AP4 induced calcium uptake. *Left:* Knockdown of TRPM1 by shRNA lentivirus decreases calcium uptake. Ratiometric imaging was done as described above. Inset shows western blots of TRPM1under different conditions. Con: mock transduced; Scr: scrambled shRNA; KD: TRPM1 shRNA. *Right:* Change in peak fluorescence ratio for all cells in each group (***p<0.05*). **D)** Inhibition of TRPM1 activity by Zn^{2+} abolishes the effect of L-AP4 on Ca^{2+} uptake. *Left:* Change in peak fluorescence ratio for all recorded cells in control and $ZnCl_2$ treatment (4, 40 and 400μM **p<0.01, ***p<0.001) following store depletion. *Middle:* Effect of Zn (40μM $ZnCl_2$) on L-AP4-induced Ca^{2+} uptake. Mean change in fluorescence ratio for 8-10 cells is shown. *Right:* Change in peak fluorescence ratio for all cells in control and $ZnCl_2$ treated cells is shown (***p<0.*001, control vs − Zn^{2+}*, p<0.01; control vs + Zn^{2+}, p<0.001; − Zn^{2+} vs + Zn^{2+}, p<0.001*).

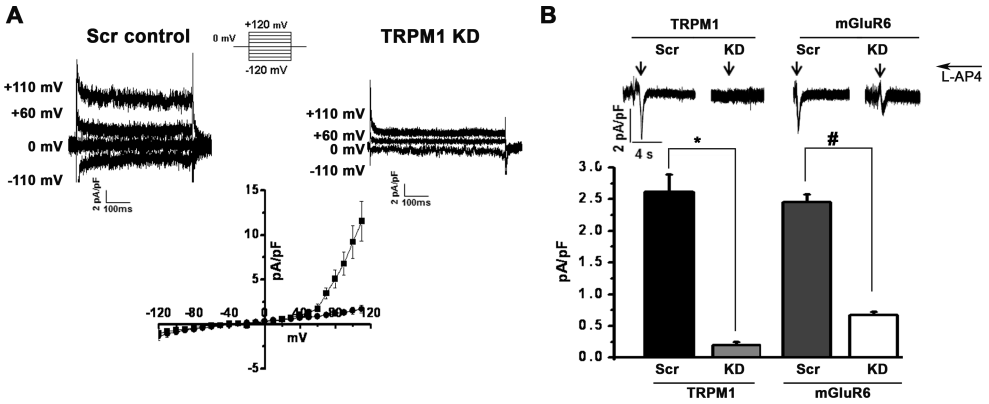

Fig. 2. Whole-cell patch clamp measurement of TRPM1 currents in human epidermal melananocytes

A) *Top:* Representative current traces from control melanocytes and TRPM1 knockdown cells at −100 mV, 0 mV, +60 mV, +110 mV potential, respectively. The IV (current-voltage) protocol is as shown in the figure inset. *Bottom:* The average TRPM1 current density plot in melanocytes (bottom); Scr (■) and TRPM1 knockdown cells (•) (n=8/each, $p<0.05$). **B)** mGluR6 modulates TRPM1 channel and activates channels opening event. *Top:* Representative current traces recorded from control (scrambled shRNA lentivirus transduced) melanocytes and cells transduced with TRPM1 and mGluR6 shRNA lentivirus. Stimulation with L-AP4 (10μM) induced an inward current in control melanocytes. Knockdown of TRPM1 abrogated the L-AP4-induced current. Similarly, mGluR6 knockdown also reduced the response to L-AP4. *Bottom:* Effect of LAP-4 stimulation on TRPM1 channel activity in melanocytes. *, # $p<0.001$; ↓ indicates L-AP4 addition. Measurements from 5-7 cells from 3 different experiments are shown ($p<0.001$).

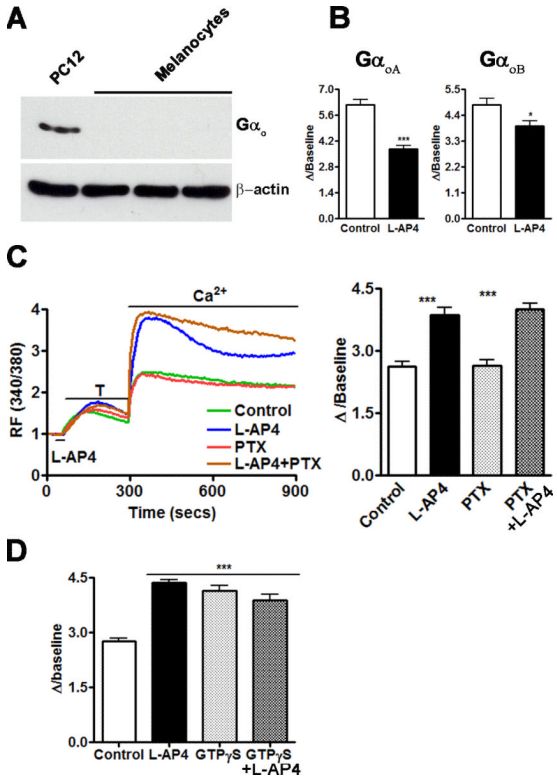

Fig. 3. Role of G proteins in L-AP4 induced Ca^{2+} uptake by melanocytes

A. Western blot for $G\alpha_o$ expression in primary melanocytes. Proteins were separated by SDS-PAGE and immunoblotted with anti-$G\alpha_o$ antibody. PC12 cells were used as positive control and β-actin levels show equal protein loading. **B)** Effect of L-AP4 on Ca^{2+} uptake by melanocytes transfected with $G\alpha_{oA}$ and $G\alpha_{oB}$. Cytoplasmic [Ca^{2+}] in Fura-2 loaded melanocytes was monitored as described in Methods. Change in peak fluorescence ratio in 8-10 cells/group is shown (****p<0.001; *p<0.05)*. **C)** Lack of involvement of PTX-sensitive G proteins in calcium uptake by melanocytes. *Left:* Ca^{2+} transients in cells cultured with or without 100ng/ml of pertussis toxin (PTX). The effect on L-AP4-induced Ca^{2+} uptake on control and PTX-treated cells was studied as described in Methods and Fig 1. *Right:* Change in peak fluorescence ratio in 8-10 cells (*p<0.001*). **D)** Requirement of GTP-binding proteins Ca^{2+} homeostasis in melanocytes. Effect of treatment with GTPγS (20μM) on intracellular calcium was measured as in Fig 1. Change in peak fluorescence ratio in 8-10 cells/group of control and treated cells is shown (*p<0.001*).

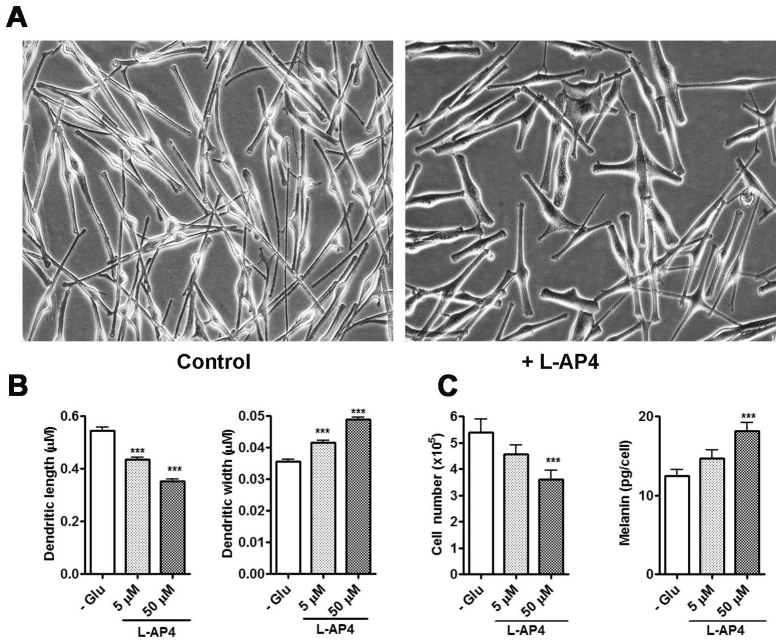

Fig. 4. Effect of L-AP4 on melanocyte morphology, growth and melanin

A) Human primary melanocytes were cultured either in the presence or absence of glutamate or L-AP4 (5-500 μM) for 6 days and photographed. Short and stubby, but wider, dendrites could be seen in L-AP4 treated cells compared to cells cultured without glutamate. **B**) Dendritic lenght (*left*) and width (*right*) were measured in >300 cells in five randomly chosen microscopic fields (***p<0.001). **C)** *Left:* Viable cells in control and L-AP4 treated melanocyte cultures were estimated using trypan blue exclusion method (***p<0.001*). *Right panel:* Alkali-soluble total cellular melanin was estimated using spectrophotometric method and as picogram melanin/cell (****p<0.001*).

Table 1

Primers for real time PCR

Gene	Accession Number	Forward Primer	Reverse Primer	Amplicon size (base pairs)
GRM 1	NM_000838	5′gcaaccaaacagccgtcat3′	5′gaatcggctgggcatcct3′	129
GRM 2	NM_000839	5′tcaacgaggccaagttcattg3′	5′ggctgactgacacgcacatg3′	132
GRM 3	NM_000840	5′cactgggcagaaaccttatcg3′	5′ccctggttgcatattcttcattt3′	120
GRM 4	NM_000841	5′cgcccagtcggtgaagata3′	5′tgtcatcctcgttggcaaag3′	119
GRM 5	NM_000842	5′gcatcatcgttgccctcttt3′	5′aactcctaggttggtggtgttacag3′	104
GRM 6	NM_000843	5′ccaagacctcacccatcttg3′	5′atgttcctgcggttgttctc3′	133
GRM 7	NM_000844	5′ctatgaccagaggcccaatga3′	5′gccaggaagacaggaatcaca3′	102
GRM 8	NM_000845	5′aaccgtatccaccgaatatttgag3′	5′gagatgaggctgaaggtgatcac3′	101
GAPDH	NM_002046	5′-gaaggtgaaggtcggagtc-3′	5′-gaagatggtgatgggatttc-3′	226

*Equine (Ec) and

**Mouse (Mm) actin;

***mouse, equine and human (Hs) GNAO1