MEMBRANE POTENTIAL REGULATED TRANSCRIPTION OF THE RESTING $K^+$ CONDUCTANCE TASK-3 VIA THE CALCINEURIN PATHWAY

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Running Title: Transcriptional Control of TASK leak $K^+$ channels

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The 2P domain $K^+$ channel TASK-3 is highly expressed in cerebellar granule neurons where it has been proposed to underlie the $K^+$ leak conductance, $I_{Kso}$. In a previous work we showed that expression of TASK-3 increases in cerebellar granule neurons as they mature in culture. Here we show that within the cerebellum, levels of TASK-3 mRNA increase as granule neurons migrate to their adult positions and receive excitatory mossy fiber input. To understand the mechanism of this increase in TASK-3 expression we used an in vitro model culturing the neurons in either depolarizing conditions mimicking neuronal activity (25K, 25 mM KCl) or in conditions which approach deafferentation (5K, 5 mM KCl). An important increase in TASK-3 mRNA is uniquely observed in 25K, and is specific since other background $K^+$ channel levels remain unchanged or decrease. The rise in TASK-3 mRNA leads to an increase in TASK-3 protein and the $I_{Kso}$ conductance resulting in hyperpolarization. Blocking L-type calcium channels or their downstream effector calcineurin, abrogates TASK-3 expression and $I_{Kso}$, leading to hyperexcitability. This is the first study demonstrating that depolarization-induced $Ca^{2+}$ entry can directly regulate cellular excitability by dynamically regulating the transcription of a resting $K^+$ conductance. The appearance of this conductance may play an important role in the transition of depolarized immature neurons to their mature hyperpolarized state during neuronal development.

Cerebellar granule neurons (CGNs) are the excitatory glutamatergic interneurons which transmit mossy fiber input to the Purkinje cells (1-3). The standing outward $K^+$ current $I_{Kso}$, plays an important role in controlling CGN excitability since it behaves as a leak conductance and is inhibited by the activation of muscarinic acetylcholine receptors (4,5). For example, in cultured rat CGNs the application of muscarine increases the input resistance and enhances cell excitability by reducing $I_{Kso}$ (4). This mechanism has also been shown to have a clear physiological role in vivo. In the vestibule-cerebellum, inhibition of $I_{Kso}$ by acetylcholine released from cholinergic mossy fibers increases the firing frequency of CGNs, which in turn enhances the spontaneous EPSCs in Purkinje neurons (6).

The molecular components of $I_{Kso}$ have been identified as TASK-1 and TASK-3, members of the tandem pore family of $K^+$ channels (7-11). TASK channels are constitutively active leak or background $K^+$ channels (7-9). They are highly sensitive to external pH, such that acidic conditions reduce while alkaline conditions enhance their activity (7-9). They are reversibly inhibited by a variety of peptide hormones and neurotransmitters acting via Gq-coupled receptors (10,12-15). CGNs express two subtypes of currents corresponding to either TASK-3 homomeric or TASK-1/-3 heteromeric channels (4,7,9). These channels are slightly different, in that they show distinct sensitivities to external pH, and only homomeric TASK-3 channels are blocked by certain agents, such as ruthenium red (19,20) and zinc (21).

It is well documented that elevated concentrations of external potassium, or other depolarizing conditions increase intracellular calcium levels and promote long term survival of CGNs in culture (22-24). It is believed that it mimics the glutamatergic innervation that CGNs receive in vivo from mossy fibers by post-natal day 12 (25). Several studies have reported that neurotransmitter or ion channel subunit expression depends on the culture conditions (26-29). For instance, levels of the GABA\textsubscript{A} receptor alpha6 subunit, the principle subunit of the cerebellum, are lower in CGNs when they are cultured in high $K^+$ versus physiological...
concentrations of K+ in the presence of growth factors that inhibit apoptosis (28,29). In contrast, the GABA_A delta subunit mRNA increases in high K+ cultures and its in vivo expression coincides with the formation of synaptic contacts of GNs with afferent excitatory mossy fibers within the IGL. These findings strongly suggest that synaptic depolarization plays a role in the expression of this subunit (28,29).

Mature neonatal cultures of CGNs express particularly high levels of TASK-3, and most of the currents in one week old cultures correspond to TASK-3 homomeric channels (17,18). Indeed, this increased expression of TASK-3 is largely responsible for K+-dependent apoptosis observed in CGNs (11,17). In the present work, we took advantage of the CGN cell culture model to understand the mechanism of TASK-3 up-regulation and determine that depolarization-induced Ca^{2+} influx is involved in the regulation of TASK-3 expression.

**Experimental Procedures**

**Cell Cultures and treatments** — Cerebellar granule neurons were prepared from 7-8-day-old Wistar rats (Charles River, France) decapitated under anesthesia with isoflurane. Cultures were prepared as previously described (17). 2 x 10^6 neurons were seeded on poly-L-lysine (50 μg/ml)-coated or Biocoat®35-mm dishes (Falcon) and cultured in Eagle’s basal medium (EMEM, Sigma) supplemented with 10% fetal bovine serum (Invitrogen), 2mM glutamine, BDNF (50 ng/ml), and 0.5% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. To prevent growth of glial cells, cytosine arabinoside (Ara-C) (10 μM) was added to the cultures one day following plating. Where indicated: 20 mM KCl (25K medium) or 20 mM NaCl (5K medium) were added 3h following plating. As indicated, nifedipine (10 μM) was added 3h following plating.

**Hippocampal and cortical neurons** were prepared from 18–19-day old fetal Wistar rats (Charles River, France). 2 x 10^6 neurons were seeded on poly-D-lysine-coated plates and cultured in EMEM supplemented with 10% horse serum (Sigma), 2mM glutamine and 0.5% penicillin-streptomycin at 37°C in a humidified environment containing 5% CO_2. To prevent growth of glial cells, cytosine arabinoside (Ara-C) (10 μM) was added to the cultures one day following plating. Where indicated: 20 mM KCl (25K) or 20 mM NaCl (5K) were added 3h following plating. As indicated, nifedipine (10 μM) was added 3h following plating.

**COS-7 cells** were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum and 0.5% penicillin-streptomycin at 37°C in a humidified environment containing 5% CO_2. Cells were transfected with rat TASK-3 or rat TASK-1 cDNAs using the DEAE-dextran method. These cells were used for either western blot analysis or immunocytochemistry.

**Total RNA Extraction, Reverse Transcription and Quantitative PCR** — Total RNAs from hippocampal, cortical and cerebellar granule neurons cultured in 35-mm dishes were extracted with the Nucleospin RNA II Kit (Macherey-Nagel). Reverse transcription was performed with 2 μg of total RNA using Superscript II reverse transcriptase (Invitrogen). Real-time PCR assays for each gene target were performed on cDNA samples using SYBR Green Mastermix Plus (Eurogentec) in 96-well optical plates on an ABI Prism 7700 Sequence Detection system (PE Biosystems). PCR data was captured using Sequence Detector Software. The data were analyzed using the comparative C_T method where the amount of target is normalized to an endogenous reference (cyclophylin D) (User Bulletin N°2 Applied Biosystems). Experiments were performed in triplicate. Primer sequences which were used for quantitative PCR are provided in supplementary information. Standard curves were generated for each set of primers using serial dilutions of rat brain cDNA to ensure a similar efficiency of amplification.

**In situ hybridization** — For in situ hybridization, post-natal day 6, 12 and 30 (P6, P12 and P30) Wistar rats were anesthetized and decapitated. Brains were removed and frozen at -40°C. Horizontal and coronal brain sections (12 μm) were cut in a cryostat (Leica, France), thaw-mounted onto charged slides (Superfrost Plus), and immediately fixed for 5 minutes in 4% phosphate-buffered paraformaldehyde, pH 7.4. Prehybridization and hybridization were performed as described previously (30,31) using identical [α-35P]-labeled sense and antisense riboprobes. After hybridization, slides were...
washed and exposed to film (BioMax, Kodak) for about 6-7 days. The resulting autoradiograms were imaged using a film scanner at 3000 dpi resolution and processed in Adobe Photoshop. Selected slides were then covered with photographic emulsion (Hypercoat RPN40, Amersham) and left to expose at 4°C for 5 weeks. After development, slides were stained with cresyl violet.

Preparation and characterization of a polyclonal rabbit TASK-3 antibody — Anti-TASK-3 antibodies were raised against a glutathione-S-transferase fusion protein containing the carboxyl-terminal 55 amino acids of mouse TASK-3 (from Thr348 to Ile402, genbank accession number NP001029048). The preparation of fusion proteins and rabbit immunization were performed as previously described (32).

Immunocytochemistry — Cerebellar granule neurons or COS transfected cells were fixed in 4% phosphate-buffered paraformaldehyde for 20 minutes, treated with 50 mM NH4Cl and permeabilized in 0.1% Triton X-100 for 5 minutes. After blocking for 1 hour in PBS containing 5% goat serum and 0.1% Tween-20, cells were incubated with TASK-3 antibody (1:500 to 1:1000) overnight at 4°C. After washes, cells were incubated in goat anti-rabbit Alexa Fluor 488 (1/800) for 1 hr at room temperature. Cells immuno-reactive for TASK-3 were visualized using an epifluorescence microscope (Axioplan 2, Carl Zeiss) equipped with 25X and 63X oil immersion objectives. Images were recorded with a cooled CCD camera (Coolsnap HQ, Photometrics) driven by Metavue software.

Electrophysiological recordings — Currents were recorded in the perforated patch configuration or in the standard whole cell configuration. The external solution contained (in mM) 120 NaCl, 5 KCl, 2 MgCl2, 0.5 CaCl2, 5 glucose, 10 HEPES, pH 7.4 with NaOH. In the depolarizing solution, 20 mM of NaCl was replaced with KCl. The perforated patch pipette solution contained 240 μg/ml amphotericin B, and (in mM) 125 KCl, 5 MgCl2, 0.1 EGTA and 5 HEPES pH 7.2 with KOH. The whole cell patch pipette solution contained (in mM) 140 KCl, 2 MgCl2, 2 K-ATP, 2.7 CaCl2, 5 EGTA and 10 mM HEPES pH 7.4 with KOH. Cells were continuously superfused with a microperfusion system during the time course of the experiments (0.1 ml/min). Experiments were performed at room temperature. Acidic pH of the external solution was obtained with HCl. CNQX stock solution (20 mM, distilled water) were stored at -20°C and used on the day of experiment. Zinc and ruthenium red (Sigma) were directly dissolved in the external medium. The synaptic activity was analyzed using Mini-analysis program (Synaptosoft, Fort Lee, USA), the threshold for detection was 15 pA.

RESULTS

During cerebellar development, CGNs initially localized in the external granule layer (EGL), migrate across the molecular layer (ML) to reach the internal granule layer (IGL). Within the IGL, GNs synapse with afferent glutamatergic mossy fibers (1-3). We have previously shown that the level of TASK-3 mRNA increases as a function of cerebellar development (17). In this work, we show that the highest increase in TASK-3 mRNA occurs in the second week after birth. The temporal expression profile of TASK3 mRNA parallels that of the GABAα alpha6 receptor subunit mRNA, a well known marker of mature CGNs (33) (Fig. 1A).

In situ hybridization analysis of rat brain slices at different developmental stages using a TASK-3 specific antisense riboprobe was used to study TASK-3 expression in detail. Little or no labeling was observed with the corresponding sense probe (data not shown). At postnatal day 7 (P7), before the migration of CGNs, a weak expression of TASK-3 was observed in the EGL (Fig. 1B). At postnatal day 12 (P12), neurons that had reached the IGL were strongly labeled compared to granule neurons remaining in the EGL (Fig. 1B-D). In adult (P30), the hybridization signal was slightly increased compared to the signal in the IGL at P12. As previously described, TASK-3 mRNA is strongly expressed in the adult cerebellum, but is also abundant in many other brain structures such as the cerebral cortex, hippocampus, striatum, and brain stem (30). Taken together, these findings show that the increase of TASK-3 mRNA levels in granule neurons coincides with their arrival in the IGL and suggest that depolarization due to synaptic activity may contribute to the increase in TASK-3 mRNA expression.

To test the hypothesis that neuronal depolarization plays a role in increasing TASK-3 expression, cultured CGNs were grown in a medium containing either a physiological (5mM, 5K medium) or depolarizing (25mM, 25K
medium) concentrations of potassium chloride (KCl). Granule neurons cultivated in 5K die by apoptosis during the first days in culture. To prevent apoptosis, BDNF was added to the culture medium. BDNF by itself did not modify TASK-3 mRNA expression in either 5K or 25K (data not shown). As shown in Fig. 2A, TASK-3 mRNA expression increases significantly in depolarizing versus physiological conditions depending on the number of days in culture (Fig. 2A). The TASK-3 mRNA levels increase as a function of external potassium concentration with maximal levels at 20 mM KCl (Fig. 2B). Since K⁺-dependent depolarization is thought to mimic glutamatergic excitatory mossy fiber stimulation in vivo (25), we also examined the effect of glutamate on TASK-3 expression. Treating the cells with 100 μM glutamate for 48 h, similarly increased TASK-3 mRNA levels to those obtained with exposure to 25 mM KCl (Fig. 2B). Given that other background K⁺ channels are also expressed in the cerebellum, we tested whether their expression was also regulated by KCl-induced depolarization. Amongst the two pore K⁺ channels tested, only the mRNA for TASK-3 is up-regulated, while the others are either down-regulated or remain unchanged (Fig. 2C). Interestingly, TASK-1, which can associate with TASK-3 to form heteromeric channels (17,19,34) was found to be significantly down-regulated by KCl.

To follow whether the rise in TASK-3 mRNA is reflected by an increase in TASK-3 protein, we generated an antibody against the carboxy terminus of mTASK-3. Anti-TASK-3 specifically detects a duplex in lysates from COS cells transfected with rTASK-3, while no bands were detected in the control conditions (Supplementary Fig. 1A). The antibody was also characterized using immunocytochemistry on COS cells transfected with the rTASK-3 subunit. Only cells expressing rTASK-3 were labeled, while cells transfected with the rTASK-1 subunit or the vector alone were not fluorescent (Supplementary Fig. 1B). Immunocytochemistry was also performed on CGNs cultured in either 5K or 25K for 7 days. A specific membrane labeling was observed for TASK-3 only under depolarizing conditions (Fig. 3A) demonstrating that the increase in TASK-3 mRNA leads to a concomitant increase in protein.

We and others have previously shown that the TASK channels underlie IKso in CGNs cultured in 25K (4,17,18). A detailed electrophysiological comparison between granule neurons cultured in 5K versus 25K was performed. Depolarizing medium significantly increases the amplitude of the pH-sensitive IKso component (29.3 ± 6.0 pA, n=25, p=0.0005) as compared to the current observed in neurons cultured in 5K (1.6 ± 0.3 pA, n=17, p=0.0005) for a period of 6-10 days (Fig. 3B/C). Conditions that increase IKso current lead to membrane hyperpolarization (Fig. 3C). The resting membrane potential measured in physiological K⁺ concentration was -40.9 ± 3.4 mV (n=13) as compared to -68.4 ± 2.9 mV in depolarizing conditions (n=28, p<10⁻⁶).

We used a pharmacological approach to distinguish the TASK-3 homomer from TASK-1 homomeric and TASK-1/-3 heteromeric channels. Zinc (100μM) and Ruthenium Red (10μM) are known to selectively inhibit TASK-3 homomers (19,21,34). Zn²⁺ (100μM) and ruthenium red (10μM) reduced the current to 25.7 ± 3.4 % (n=7, p=0.001) and 23.1 ± 2.6 % (n=8, p=0.0006) respectively, which is the same degree of inhibition observed with acidosis (pH 5.5) which was 16.7 ± 2.3 % (n=10, p<10⁻⁴) (Fig. 3D). The application of Zn²⁺ and ruthenium red leads to membrane depolarization (25K: -73.6 ± 4.2 mV, n=7; 25K pH 5.5 : -35.9 ± 2.5 mV, n=7, p<10⁻⁶; 25K + Zn²⁺: -51.9 ± 4.3 mV, n=7, p=0.0002; 25K+ RR: -48.8 ± 6.2 mV, n=5, p=0.0005). These results confirm that the TASK-3 homomeric channel is the principle component of IKso recorded in 25K. The difference observed between current inhibition obtained with external acidosis and that with Zn²⁺/RR could be due to TASK-1 homomers and/or TASK -1/-3 heteromers, since both channels are resistant to inhibition by Zn²⁺ and RR. Indeed, PCR quantification experiments reveal that TASK-3 mRNA levels strongly increase in 25K, while levels of TASK-1 mRNAs decline, which might explain the low levels of TASK-1/-3 heteromeric channels and/or TASK-1 homomeric channels under such culture conditions.

Potassium depolarization of granule neurons leads to the activation of the L-type voltage dependent calcium channel (VDCC) (24,35). To establish whether calcium entry via VDCC is responsible for the increase in TASK-3 expression, we used the L-type inhibitor nifedipine. Nifedipine totally blocks the depolarization-induced increase in TASK-3 mRNA (Fig. 4A), suggesting a correlation between an increase in intracellular calcium and TASK-3 mRNA levels. Increases in intracellular
Ca\(^{2+}\) lead to the activation of calcineurin, a Ca\(^{2+}/\) calmodulin-dependent protein phosphatase, which is crucial for the regulation of the expression of numerous genes in CGNs (36,37). To test whether this phosphatase plays a role in the expression of TASK-3, granule cells were cultured under depolarizing conditions in the presence or absence of FK506 (2.5 \(\mu\)g/ml) and cyclosporin A (1 \(\mu\)M), two inhibitors of calcineurin (38). FK506 and cyclosporin A prevent the depolarization-induced increase in TASK-3 mRNA (Fig. 4A). Moreover, the TASK-3 protein and consequently IKso are also strongly reduced by these calcineurin inhibitors (Fig. 4B/C). Acute application of FK506 on neurons cultured in 25K has no immediate effect on IKso (data not shown). KN93, an inhibitor of CaM kinases which are also downstream effectors of Ca\(^{2+}\) signaling (39), was without effect on either TASK-3 mRNA or protein in 25K (Fig. 4A/B). Furthermore, cells grown in 25K with FK506 display depolarized resting membrane potentials with values close to those recorded in cells grown in 5K conditions (Fig. 4C - right panel) indicating that FK506 is acting at the level of TASK-3 expression to decrease IKso.

Ca\(^{2+}\)-dependent TASK-3 expression could occur at the transcriptional and/or the post-transcriptional level. To establish whether transcriptional activity is required for the up-regulation of TASK-3, cells were incubated for 6 or 12 hours in the presence of actinomycin D, an inhibitor of mRNA synthesis. Actinomycin D prevents the increase in TASK-3 mRNA which occurs when cell cultures are switched from 5K to 25K (Fig. 5A). In contrast, under the same conditions, the protein synthesis inhibitor cycloheximide, fails to block this up-regulation suggesting that de novo protein synthesis is not required for Ca\(^{2+}\)-dependent TASK-3 mRNA up-regulation (Fig. 5B). While, application of cycloheximide does lead to a decrease in TASK-3 mRNA levels (Fig. 5B), this occurs in both 5K and 25K, suggesting that de novo protein synthesis is necessary for stabilizing the messenger, but in a calcineurin-independent manner (Fig. 5B).

To determine the kinetics of the response of TASK-3 mRNA expression, 48 hour old cultures grown in 5K were either maintained in 5K or switched to 25K. A significant increase in TASK-3 mRNA levels is observed within 6h of KCl-induced depolarization (Fig. 6A). However, a constant depolarization is required to maintain the level of TASK-3 transcripts, since cultured CGNs which were maintained for 48 hours in 25K medium and subsequently switched to 5K medium for 3, 6 or 9h (Fig. 6B) show a rapid decrease in TASK-3 transcripts. The level of the TASK-3 transcript in switched cultures for 6h and 9h was 76 ± 20% and 31 ± 10% of that observed in control cultures maintained in 25K medium, respectively (p<0.01). These results demonstrate that cultured CGNs require the continued presence of depolarizing conditions to maintain expression of the TASK-3 subunit mRNA and that this regulation is dynamic.

As mentioned above, cells grown in 25K, i.e. expressing high levels of TASK-3, are more hyperpolarized (about -70 mV for 8 DIV), than cells grown in the presence of FK506 (-40mV) (Fig. 4C) or cells grown in 5K (-40mV) (see Fig. 3C), strongly suggesting a direct control of the resting membrane potential by IKso/TASK-3. Since CGNs, particularly rat GNs compared to mouse GNs, grown in high potassium are electrically silent (29,40,41) and display hyperpolarized resting potentials (4,17,29,42), we analyzed the influence of IKso/TASK-3 expression on the synaptic activity of the GNs. The current of a single neuron was recorded at -60 mV in the whole cell configuration to follow glutamate receptor activation. An acute perfusion of a K\(^+\)-rich solution was applied to the neurons surrounding the patched neuron and the excitatory post synaptic currents (EPSCs) were recorded. Only the neuron cultivated in 25K+FK506 presents a statistically significant large induced synaptic activity which is sensitive to the AMPA receptor blocker CNQX (Fig. 7A). The mean induced activities in 25K and in 25K+FK506 were significantly different (t test: p=0.041) (Fig. 7B). In the 25K+FK506 condition the induced activity is significantly different in the presence and absence of CNQX (t test; p=4.4 \(10^{-4}\), n=4), which is not the case for the 25K condition (t test; p=0.16, n=7). These findings strongly suggest that the highly increased excitability of granule neurons grown in the presence of FK506 is explained by a lower expression of TASK-3 and show the important impact of IKso expression within these neurons.

To study whether other neuronal subtypes expressing the TASK-3 mRNA are similarly sensitive to K\(^+\)-induced depolarization, we quantified the mRNA levels in fetal cultures from either hippocampus or cortex grown in high potassium for 48 hrs in the absence or presence
of nifedipine (Fig. 8). Both neuronal subtypes show a nifedipine-sensitive increase in TASK-3 transcripts. However, the level of Ca\(^{2+}\)-induced TASK-3 transcript is significantly lower than that observed in cerebellar granule neurons, approaching levels observed in 5K. Nevertheless, these findings demonstrate that transcriptional regulation of TASK-3 by intracellular Ca\(^{2+}\) is not unique to CGNs, and suggest that this regulation may be an important mechanism throughout the brain.

**DISCUSSION**

Since the resting K\(^+\) conductance IKso, is largely encoded by TASK-3 (17,18), we wanted to determine how TASK-3 expression increases as a function of neuronal maturity. Because neuronal maturity is achieved by culturing CGNs in depolarizing conditions (25), we compared TASK-3 expression in conditions mimicking deafferentation (physiological K\(^+\) / BDNF) to conditions mimicking neuronal activity (high concentrations of K\(^+\) / BDNF). This study shows a central role for depolarization induced Ca\(^{2+}\) influx in the regulation of TASK-3 expression in cultured CGNs. Changes in the level of TASK-3 mRNA in response to depolarizing stimuli paralleled those observed during granule neuron development in the cerebellum in which a high increase in mRNA levels coincides with the formation of synaptic contacts between GNs and excitatory mossy fibers. It has been observed in numerous neuronal cell types including CGNs, that the resting membrane potential shifts from a relatively depolarized state to a more hyperpolarized state during maturation (24). Thus, the results of our study suggest that the depolarization dependent expression of TASK-3 might play an important role in this transition.

Our studies show that K\(^+\)-induced elevation in intracellular calcium mediates the increase in transcription of TASK-3 mRNA. Nifedipine, a blocker of L-type calcium channels, prevents the increase in TASK-3 mRNA levels, suggesting that calcium entry via these channels is involved in regulating channel expression. Moreover, we show a key role for the Ca\(^{2+}\)/calmodulin dependent phosphatase, calcineurin. The two calcineurin inhibitors FK506 and cyclosporin A, completely abolish the increase in TASK-3 mRNA, whereas an inhibitor of CaM kinases, KN93, is without effect. In CGNs, calcineurin has already been shown to play a fundamental role in the regulation of numerous genes (36,37). While activation of calcineurin regulates genes implicated in the processes of cell proliferation, differentiation, migration and neurite growth of immature granule cells in the EGL, inactivation of calcineurin is thought to predominantly up-regulate genes implicated in synaptic transmission in mature granule cells in the IGL (for review see (24)). This appears not to be the case for TASK-3, since its expression increases when calcineurin is active, and as neurons mature. One of the best studied calcineurin targets is nuclear factor of activated T cell (NFAT), a transcription factor that when dephosphorylated by calcineurin, translocates to the nucleus to activate gene expression (43). Recent studies suggest that calcineurin may also act on another important transcription regulator myocyte enhancer factor 2 (MEF2) that is also expressed in CGNs. MEF2 binds to DNA in its hypophosphorylated form and calcineurin enhances the MEF2 binding activity by maintaining the protein in this state (44-46). However, in our hands, dominant-positive forms of NFAT (47) and MEF2 (48) were unable to increase TASK-3 expression in CGNs (data not shown). Future studies will be required to determine exactly how calcineurin regulates TASK-3 expression.

Interestingly, the expression of both TASK-1 and TASK-3 mRNAs is regulated by calcium. Whereas the level of TASK-3 mRNA is increased by calcium, the level of TASK-1 mRNA is reduced. These findings may explain the differences observed between CGNs grown in high K\(^+\) (10,17,18) and cultures grown in physiological concentrations of K\(^+\) in the presence of growth factors and after a short stimulation of glutamate (42,49). In the latter papers both homomeric TASK-3 channels and heteromeric TASK-1/-3 channels contribute to IKso. In high K\(^+\), TASK-3 homomers are largely the main contributors to IKso, in accordance with an increased TASK-3 and a decreased TASK-1 mRNA expression. The calcium-dependent transcriptional regulation of these channel subunits would give CGNs the facility to regulate the relative levels of hetero- and homomeric channels with their distinct sensitivities to pH changes and neurotransmitter responses (19). While, the temporal profile of TASK-3 mRNA expression in CGNs in vitro follows that observed in vivo, this is not the case for TASK-1. TASK-1 mRNA is reduced in
neurons cultured in high K⁺, while it is increased in vivo during cerebellar development (17,50). A similar situation is observed with the GABAₐ receptor (51). The expression of both the alpha1 and alpha6 receptor subunits increase during cerebellar development in parallel with the establishment of synaptic contacts in the internal granule layer. However, K⁺-induced calcium entry in cultured neurons only increases the alpha1 subunit mRNA (51).

The calcineurin inhibitor FK506 potently blocks TASK-3 transcription and as a consequence diminishes IKso. Since IKso is a key determinant of CGN excitability (4), the electrical properties of CGNs grown in culture conditions favoring expression of high levels of TASK-3 were compared to those grown in the presence of FK506. 8-9 DIV CGNs display hyperpolarized resting membrane potentials (about -70mV) and are electrically silent. It requires a depolarizing stimulus (see Experimental Procedures), for some CNQX-sensitive EPSCs to be recorded. On the other hand, cells grown in high K⁺ and FK506 are more depolarized (about -40mV), and are highly sensitive to a depolarizing stimulus. Taken together, these results demonstrate a close correlation between the Ca²⁺-dependent activation of calcineurin and the expression of IKso leading to a change in CGN excitability. Indeed, the importance of IKso in adaptive regulation of excitability, was divulged in a knockout mouse model of the GABAₐ α6 receptor subunit (50), wherein the removal of a tonic inhibitory conductance leads to a long-term increase in the leak conductance IKso (50). Interestingly, recent work on a mouse knock-out model of TASK-1, demonstrated that the lack of TASK-1 did not affect the resting membrane potential of CGNs and that the IKso conductance was carried by TASK-3 homomeric channels (18). In the future it will be important to determine the electrical properties of CGNs derived from TASK-3 and TASK-1/TASK-3 knock-out mice.

The physiological importance of TASK-like background activity has clearly been demonstrated in several neuronal subtypes besides cerebellar granule neurons, such as thalamocortical neurons (52), hypoglossal motoneurons (53), brainstem motoneurons (54) and serotonergic raphe neurons (31). For example, in thalamocortical relay neurons, TASK channels control activity modes during the sleep-wake cycle (52). In motoneurons and aminergic brainstem neurons, the immobilizing and soporific effects of anesthetics have been attributed to anesthetic activation of TASK channels (54-56). In serotonergic raphe neurons, the pH-dependent inhibition of IKso channels has been suggested to contribute to ventilatory and arousal reflexes associated with extracellular acidosis (31). TASK channels are also thought to underlie the oxygen-sensitive background K⁺ channel in the chemoreceptor carotid body Type I cells (57,58). Hypoxic inhibition of TASK currents and subsequent depolarization of these oxygen-sensing cells signals the respiratory center in the brainstem, leading to a reflex increase in breathing (58). In all cases, the fine tuning of these channels are key elements in the regulation of neuronal excitability.

Our work reveals a new and important mechanism for the control of the leak K⁺ conductance IKso. This is the first study demonstrating that depolarization-induced Ca²⁺ entry can, via calcineurin, directly regulate cellular excitability by dynamically regulating the transcription of a resting K⁺ conductance. Future work will determine what significance Ca²⁺-dependent transcriptional regulation of TASK-3 has in vivo.

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**FOOTNOTES**

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1The abbreviations used are: CGN, cerebellar granule neurons; EGL, external granule layer; IGL, internal granule layer; EPSC, excitatory post synaptic current; RR, ruthenium red; VDCC, voltage dependent calcium channel; NFAT, nuclear factor of activated T cell; MEF-2, myocyte enhancer factor 2; GABA, gamma-aminobutyric acid; DIV, days in vitro, BDNF, brain derived neurotrophic factor.

**FIGURE LEGENDS**

**Fig. 1.** The expression of TASK-3 mRNA increases in the developing rat cerebellum. A, Quantitative PCR analysis of TASK-3 mRNA expressed in the developing cerebellum. RNAs were isolated from 7, 14 and 21 day old rat cerebella, and TASK-3 mRNA levels were assessed by quantitative PCR. The temporal profile of TASK-3 expression was compared to the expression profile of GABA A alpha6, a well known marker of CGN maturation. Data were normalized to the level of cyclophilin D. Each data point represents the mean ± SEM of 3 experiments. B, Transversal brain sections from postnatal day 7, 12 and 30 rats (P7, P12 and P30) were hybridized with α [33P]-labeled cRNA probe for TASK-3 and exposed to autoradiographic film. C, Cerebellar slice from a P12 rat : high resolution images of the autoradiographic film and images of the corresponding brain slice after histological staining with cresyl violet. D, Photomicrographs of a P12 liquid emulsion coated and counterstained cerebellar section illustrating the EGL (left) and IGL layer (right). Scale bar : in C = 400 μM; in D= 15 μM.

**Fig. 2.** KCl-induced depolarization specifically increases TASK-3 mRNA in CGNs. A, CGNs were maintained in 5K or 25K for 0, 2, 4, 6 or 8 days and the corresponding RNAs were isolated. TASK-3 mRNA levels were assessed by quantitative PCR and data were normalized to the level of cyclophilin D. Each data point represents the mean ± SEM of nine experiments. B, CGNs were maintained for 2 days in a 5 to 50 mM KCl gradient (5K, 10K, 15K, 20K, 25K or 50K) or 5 mM KCl containing 100 μM glutamate. RNAs were isolated at day 2 from cultures maintained in each condition. The TASK-3 mRNA levels were assessed by quantitative PCR and data were normalized as in A. Each data point represents the mean ± SEM of nine experiments * p < 0.01 in comparison to the TASK-3 mRNA level in 5K. C, CGNs were maintained in 5K or 25K for 6 days. RNAs were isolated at day 6 from cultures maintained in each condition. TASK-3, TASK-1, TREK-1, TREK-2 and TWIK-1 mRNA levels were assessed by quantitative PCR and data were normalized as in A-C. Data were plotted as a ratio of 25K to 5K. Each data point represents the mean ± SEM of nine experiments.
Fig. 3. KCl-induced depolarization increases TASK-3 protein levels and the IKso conductance in CGNs. A, CGNs were maintained in 5K or 25K for 7 days. Immunocytochemistry was performed using a rabbit polyclonal TASK-3 antibody (which was characterized in this paper) and a Alexa fluor 488 secondary antibody. B, Whole cell currents elicited by voltage ramps from 0 to -100 mV, 1s in duration, from a holding potential of 0 mV. Neurons were cultivated in 5 mM K+ or in 25 mM K+ and always recorded in 5 mM K+ external solution. An acidic solution at pH 5.5 was applied and washed out as indicated. C, Means of the proton-sensitive currents recorded at 0 mV in the same conditions as in B. Currents were normalized to the capacitance of the neurons. The resting membrane potentials were measured under zero current clamp at the beginning of each path-clamp experiment. Number of tested neurons are indicated. D, Current and membrane potential recorded upon perfusion of acidic solution pH 5.5, zinc (100µM) and ruthenium red (RR, 10µM) in neurons cultivated in 25K.

Fig. 4. The depolarization-induced increase in TASK-3 mRNA expression depends on Ca2+-induced activation of calcineurin. A, CGNs were maintained in 5K or 25K in the presence or absence of nifedipine (Nife), FK506, cyclosporin A (CsA) or KN93. RNAs were isolated at day 2 from cultures maintained in each condition. The TASK-3 mRNA levels were assessed by quantitative PCR and data were normalized to the level of cyclophilin D. Each data point represents the mean ± SEM of nine experiments. * p < 0.01 in comparison to the TASK-3 mRNA level in 25K control. B, CGNs were maintained in 25K, 25K+FK506, 25K+KN93 and 25K+CsA. Immunocytochemistry was performed at day 7 using the TASK-3 primary antibody and a Alexa fluor 488 secondary antibody. C, Neurons were cultivated in 25 mM K+ or in 25 mM K+ with FK506 and recorded at 6 to 10 DIV. Means of the proton-sensitive currents recorded at 0 mV in the same conditions as in Fig. 3. Currents were normalized to the capacitance of the neurons. The resting membrane potentials were measured under zero current clamp at the beginning of each patch-clamp experiment. Number of tested neurons is indicated.

Fig. 5. Transcriptional activity, but not de novo protein synthesis, is required for the depolarization-induced increase in TASK-3 mRNA. A, Cerebellar granule neurons were maintained in 5K or 25K in the absence or presence of Actinomycin D (actinoD) for 6 or 12 hours. RNA was isolated at designated times from cultures maintained in each condition. TASK-3 mRNA level was assessed by quantitative PCR and data were normalized to the level of cyclophilin D. Each data point represents the mean ± SEM of nine experiments. * p < 0.01 in comparison with the TASK-3 mRNA level in 25K control. B, Cerebellar granule neurons were maintained in 5K or 25K in the absence or presence of cycloheximide (cyclo). RNA was isolated at designated times from cultures maintained in each condition. TASK-3 mRNA level was assessed by quantitative PCR and data were normalized to the level of cyclophilin D. Each data point represents the mean ± SEM of nine experiments. * p < 0.01 in comparison with the corresponding 5K TASK-3 mRNA level.

Fig. 6. KCl depolarization rapidly induces TASK-3 mRNA expression and is necessary to maintain high mRNA levels. A, CGNs were maintained for 2 days in 5K and switched to 5K or 25K for 3, 6 or 9 hours. RNAs were isolated at designated times from cultures maintained in each condition. The TASK-3 mRNA levels were assessed by quantitative PCR and data were normalized to the level of cyclophilin D. Each data point represents the mean ± SEM of nine experiments. * p < 0.01 in comparison with the corresponding data point in 5K. B, CGNs were maintained for 2 days in 25K and switched to 25K or 5K for 3, 6 or 9 hours and the corresponding RNAs were isolated. The TASK-3 mRNA levels were assessed by quantitative PCR and data were normalized as described in A. Each data point represents the mean ± SEM of nine experiments. * p < 0.01 in comparison to the corresponding data points in 25K.

Fig. 7. Influence of TASK-3 expression on neuronal excitability. A, Left panel, current recorded in whole cell configuration, at -60mV, upon acute perfusion with a depolarizing solution (depol.) in single neurons cultured in 25 mM K+ (25K) or 25K+FK506, as indicated. A, Right panel; same experiment with CNQX, in the same two neurons. B, Means of the induced synaptic activity in 25K
with or without FK506 and CNQX (number of EPSCs over 15 pA recorded in depolarizing solution for 10s minus those recorded in control condition).

**Fig. 8.** KCl-induced depolarization increases TASK-3 mRNA in fetal rat hippocampal and cortical neurons. Neurons were isolated from fetal rat hippocampus and cortex and maintained in 5K, 25K or 25K+ nifedipine for 48 hours. The corresponding RNAs were isolated and TASK-3 mRNA levels were assessed by quantitative PCR. Data were normalized to the level of cyclophilin D. Each data point represents the mean ± SEM of 3 experiments. * $p < 0.01$ in comparison with the TASK-3 mRNA level in 25K.
Figure 1

(A) Relative mRNA levels of TASK-3 and GABA alpha6 over time (P8, P13, P15, P21).

(B) Images showing brain sections at different ages (P7, P12, P30).

(C) Magnified images highlighting EGL and IGL areas.

(D) Close-up views of EGL and IGL regions.
Figure 3

A

25K

5K

B

25K

I (pA)

count

wash

pH5.5

5K

count

wash

pH5.5

V (mV)

C

Proton inhibited current (pA/pF)

V resting (mV)

5K 25K

D

I (pA)

V resting (mV)

control

tpH5.5

Zn2+ 100µM

RR 10µM

10 7 7 8

10 7 7 5

10 7 7 8

10 7 7 8

10 7 7 8

10 7 7 8

10 7 7 8
Figure 4

A

![Relative mRNA level for TASK-3](image)

B

![Immunofluorescent images](image)

C

![Proton inhibited current](image)
Figure 5

A

relative TASK-3 mRNA level

control actinoD control actinoD

6h 12h

5K 25K

B

relative TASK-3 mRNA level

control cyclo control cyclo

6h 12h

3.19 2.98 3.47 3.31
Figure 6

A

B

relative mRNA level

3h 6h 9h

TASK-3

5K → 25K

5K → 5K

25K → 25K

25K → 5K

*
Figure 7

A

25K

depol.
depol. + CNQX

25K + FK506

50 pA
5 s

B

Induced synaptic activity (number of epsc)

25K + CNQX

25K + FK506 + CNQX

ns
4
7

16
Figure 8
Membrane potential regulated transcription of the resting K+ conductance TASK-3 via the calcineurin pathway

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