PAIN PERCEPTION IN MICE LACKING THE β3 SUBUNIT OF VOLTAGE-ACTIVATED CALCIUM CHANNELS

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SUMMARY

The importance of voltage-activated calcium channels in pain processing has been suggested by the spinal antinociceptive action of blockers of N- and P/Q-type calcium channels as well as by gene targeting of the α1B subunit (N-type). The accessory β3 subunits of calcium channels are preferentially associated with the α1B subunit in neurones. Here we show that deletion of the β3 subunit by gene targeting affects strongly the pain processing of mutant mice. We pinpoint this defect in the pain-related behaviour and ascending pain pathways of the spinal cord in vivo and at the level of calcium channel currents and proteins in single dorsal root ganglion neurones in vitro. The pain induced by chemical inflammation is preferentially damped by deletion of β3 subunits, whereas responses to acute thermal and mechanical harmful stimuli are reduced moderately or not at all, respectively. The defect results in a weak wind-up of spinal cord activity during intense afferent nerve stimulation. The molecular mechanism responsible for the phenotype was traced to low expression of N-type calcium channels (α1B) and functional alterations of calcium channel currents in neurones projecting to the spinal cord.
INTRODUCTION

Voltage activated calcium channels represent a family of ionic channels that play a crucial role in the nervous system by controlling membrane excitability and neurotransmitter release. Neurones express L-, N-, P/Q- and R-type calcium channels, which are composed of the pore forming subunit α1, and the accessory subunits β, α2δ (1, 2) and probably γ (3). The importance of α1D (L-type), α1B (N-type), α1A (P/Q-type) and α1E (probably R-type) for the function of the nervous system in vivo is underlined by recent studies with genetically engineered mice (4). By contrast, the in vivo function of the four known β subunits (β1, β2, β3, β4) of calcium channels is less understood. In heterologous expression systems, β subunits are required for the functional expression of α1 subunits (5-7), enhance the current density and shift the voltage dependence of the activation and inactivation of recombinant calcium channels (8-11). Additionally, β subunits have been implicated in the modulation of calcium channels by G proteins (12-14). In vivo, the expression of β subunits is modified in pathological states such as cardiac disfunction (15) and diabetes (16). A natural occurring mutation of β4 is associated with ataxia and absence seizures in lethargic (lh/lh) mice and this mutation modifies N- and P/Q-type calcium channels in the brain (17). The targeted disruption of β1 is lethal (18). In sympathetic neurones of β3 deficient mice, N- and L-type calcium channel currents are reduced, the activation of P/Q type calcium channels is altered and no difference appears concerning inhibitory effects of norepinephrine on calcium channel currents (19). Such alterations of calcium channel currents in sympathetic neurones might be related to the cardiovascular phenotype of mice lacking the β3 subunit (20). Yet, the in vivo functions of the β3 subunit of voltage activated calcium channels are not well established.

As the α1B subunit of N-type calcium channels is primarily associated with the β3 subunit in neurones (21, 22), an altered neurotransmitter release at synaptic sites may be expected in the β3 deficient mice. Specifically, blockade of N-type calcium channels inhibits tachykinin release from afferent sensory nerves (23). Within the spinal cord, N-type calcium channels show the highest density in the superficial laminae (24, 25). Accordingly, deletion of the α1B subunit (CaV 2.2; 26-28) and intrathecal injection of the N-type channel blocker, ω-conotoxin GVIA (29), reduce the behaviour associated with pain in rodents. Furthermore, the β3 subunit might also associate with α1A subunits to form P/Q-type calcium channels,
which are present in the deeper laminae of the spinal cord (30) and might be involved in nociceptive processing (29). In the present study, we examine the possible function of β3 subunits in nociception and sensory processing using a mouse model, in which the β3 subunit has genetically been deleted. Since dorsal root ganglions (DRG) contain the cell bodies of afferent sensory fibres and predominantly express N-type calcium channel currents (31), binding assays and pharmacological testing on DRG neurones with the selective marker of N-type channels, ω-conotoxin GVIA, were used to determine the expression of α1B in DRG neurones. We have also explored for changes in the voltage dependence and G protein modulation of calcium channel currents in isolated DRG neurones. In vivo, we analysed the pain related behaviour and the spinal cord response to intense and persistent barrage of afferent nociceptive impulses (wind-up).
EXPERIMENTAL PROCEDURES

Generation of β3+/− mice. The organization of the β3 gene (32) and the targeting vector used to generate null mutations of the β3 gene are shown in Fig. 1. Linearized targeting constructs were electroporated into R1 embryonic stem (ES) cells and the cells were selected with G418 and ganciclovir. We identified 3 out of 470 ES cell clones with predicted genomic structures for the targeting vector IIMB. Selected ES cell clones were microinjected into C57BL/6 blastocysts and transferred into the uteri of pseudopregnant recipient females. Two independent ES cell clones were transmitted through the germ line. Mice were kept in essentially specific pathogen-free environment.

Construction and purification of glutathione-S-transferase (GST)-β1b fusion protein and generation of the antibodies against β1b. Nucleotides 1269-1857 (aa 403-597) of β1b (33) were amplified by PCR using the forward primer, 5′-CGG GAT CCG AGT ACT TGG AAG CCT ACT G and the reverse primer, 5′-CGG AAT TCT CAG CGG ATG TAG ACG CCT T. The amplified cDNA was ligated into the BamHI/EcoRI sites of pGEX-4T2 (Amersham Pharmacia biotech). The accuracy was confirmed by sequencing both strands. BL21(DE3) E.coli were transformed with pGEX-4AT2-β1b and protein expression was carried out as previously described (34). A synthetic peptide (K584NELEGWGQGVYIR597) of β1b (33) was coupled to keyhole limpet hemocyanin for injection into rabbit 234. Antibody 234 recognizes the GST-β1b fusion protein (Mr ∼ 59,000) and native β1b in brain and skeletal muscle (Mr ∼ 72,000, Fig. 1b). It does not recognize a GST-β1a fusion protein (Mr ∼ 33,000) or native β1a (Mr ∼ 52,000), which is predominantly expressed in skeletal muscle. Other antibodies have been described previously (22). Immunoblots were repeated up to six times. Pooled tissue from up to 3 animals (brain microsomes) and up to 20 animals (DRGs) per genotype, respectively, were processed. The data shown (Fig. 1D-E) represent independent, non-stripped and non-reused blots.

Nociceptive tests and rotord test. The test for nociception were performed as described (35). The tail flick response was evoked by a light beam (irradiated heat) or by immersing two thirds of the tail in water at 52°C. In the hot plate test, mice were placed on a plate (56°C) and responses counted when the mouse first licked the hind paws and jumped from the plate (cut-off time, 30s and 60s, respectively). Formalin (25 µl; 2% paraformaldehyde in PBS) was injected into the plantar surface of the left hind paw. The time spent licking or biting the injected paw was
recorded as the nociceptive score. Inflammation was induced by subcutaneous injection of 10 µl
of complete Freund's adjuvant (CFA, 0.5 mg/ml Mycobacterium butyricum in paraffin oil and
emulsifying agent) into the plantar surface of the left hind paw under short anaesthesia with
dimethyether. Before and 60 h after the CFA injection, the animals were placed on a raised
grid and mechanical thresholds were measured using calibrated von Frey hairs. In the rotorod
test, mice were trained on the rod for 1 min at rest, 5 min at 16 rpm, 1 min at rest and 5 min at 24
rpm. For the test, mice were placed on the apparatus facing away from the experimenter and in
the direction opposite to the rotating rod while it was moving at 32 rpm (cut off time 5 min). The
tests were performed with adult male mice. The genotype of the animals was unknown to the
investigator during the tests.

Extracellular recordings in the spinal cord. Adult male mice (30 - 40 g) of both genotypes
were used for extracellular unit recordings. The anaesthesia was induced with dimethyether
and subsequently with pentobarbital (i.p. 0.5 mg/10 g body weight). In order to control the
deep of anaesthesia, the tail vein was canulated and pentobarbital was given in form of
intravenous bolus (0.05 mg/10 g body weight) every 20 - 25 min during the experiment. A
laminectomy was performed to exposed the spinal segments Th8 - Th10 and the thoracic
vertebras were fixed with spinal hooks in a stereotaxic frame. The left sural nerve was
exposed and placed on a bipolar electrode for stimulation (Grass). The experiments were
started after a recovery period of 30 - 45 min. Extracellular recordings were made with
tungsten microelectrodes of 5 MΩ resistance (WPI, Berlin) contralateral to the stimulated
nerve (i.e. in the right spinal cord). Initially, the microelectrode was placed 350 - 500 µm
lateral to the median dorsal spinal vein in the spinal segment Th8 and carefully inserted just
under the surface. To search for responsive units, the electrode was driven from this zero
point deep through the spinal cord and trains of 50 V pulses (5 ms long) were regularly
delivered to the sural nerve. We used a frequency of 0.2 pulses per second (PPS) in this
searching phase in order to prevent wind-up. If the test pulses did not evoke spikes within
driving distances of maximally 250 µm, the electrode was removed and the searching phase
was reinitiated from a new zero point caudal to previous one. When the test pulses evoked
spikes, we first rule out the contribution of receptive fields by stimulating mechanically
(brush, pinch) the skin of the back and hind paws on both ipsilateral and contralateral sides
of recording. Since the sural nerve was cut distal to the bipolar electrode, responsive units
were used for further analysis when no response to cutaneous stimulation was observed. The
thresholds were determined with series of 12 pulses of amplitudes between 10 V and 100 V
The wind-up was finally evoked with 50 V pulses (5 ms long) delivered for 16 sec at 1 PPS. Data were collected and analysed using the Chart software for the PowerLab system (ADInstruments).

Isolation of DRG neurones. Dorsal root ganglions (DRG) were obtained from adult wild type and β3-/- mice and neurons were dissociated by incubation in the presence of trypsin type I (0.67 mg/ml medium; Sigma, Deisenhofen) and collagenase type II (4 mg/ml medium; Biochrom Berlin) for 20 min. The cell suspension was centrifuged for 5 min, the supernatant withdrawn and DMEM medium supplemented by 10% foetal calf serum was added. Isolated neurones were plated onto poly-L-lysine (Sigma) coated glass cover slips and kept in an incubator. Electrophysiological experiments were performed within 4-16h after dissociation.

Whole-cell patch clamp. For electrophysiological recordings glass cover slips were placed into a recording chamber and mounted on an inverted microscope. Cells were perfused with extracellular solution and electrophysiological recordings were started after 10 min. DRG neurons were identified based on their typical morphology using a 40x phase contrast objective. Ba²⁺ currents (I_Ba) from wild type and β3-/- DRG neurons were recorded using standard whole cell recording techniques (36). I_Ba was recorded with an EPC-9 amplifier (Heka). Upon establishment of the whole cell configuration, series resistance, fast and slow capacitance components were compensated using the internal circuit of the EPC-9 amplifier. The currents were leak subtracted by applying n/4 correction mode. Data were sampled at 10 kHz, filtered at 3.3 kHz. Data were analysed off line using the Pulse-Fit (Heka) software package. For further analysis, current traces were imported into Sigmaplot (Jandel Scientific). For display current traces were transferred to Corel draw (Corel Graphics). All patch-clamp experiments were performed at room temperature. Pipettes of 2-3 MΩ resistance were prepared on a DMZ Universal Puller from 1.5 mm borosilicate glass capillaries (Clark Electromedical Instruments). The composition of the recording solutions used was the following (in mM): Intracellular solution, CsCl 95, CsSO₄ 40, TEA-Cl 20, CaCl₂ 1, EGTA 10, Mg-ATP 3, Hapes 10 pH 7.3 (CsOH); extracellular solution, TEA-Cl 135, BaCl₂ 10, MgCl₂ 1.2, Hapes 10, pH 7.4 (TEA-OH).

Conotoxin binding assays. Whole dorsal root ganglions were isolated and homogenized in 5 volumes of 5 mM TRIS-HCl, pH 7.4 containing a protease inhibitor mix (0.1 mM phenylmethylsulfonyl fluoride, 1mM phenanthroline, 1mM iodoacetamide, 1mM benzamidine,
1μM pepstatin A, 1μg/ml antipain and 1μg/ml leupeptin). Equilibrium binding of $^{[125I]}\omega$-conotoxin GVIA (Amersham Pharmacia Biotech, Freiburg) in the absence and presence of 10 nM unlabeled ω-conotoxin GVIA was performed in duplicate at a total incubation volume of 500 μl containing 10 μg of protein. Binding to isolated DRG neurones was performed as follows: intact neurones were incubated in the presence of 50 pM $^{[125I]}\omega$-conotoxin GVIA for 2 hours, then washed three times with DMEM containing 0.1% BSA. Non-specific binding measured in the presence of 200 nM unlabeled ω-conotoxin GVIA was less than 20% of total binding. Omega-Conotoxin GVIA-binding has been quantified by non-linear regression analysis using the statistical package for social sciences (SPSS) software.
RESULTS

Targeted disruption of the β3 gene

The β3 gene (Fig. 1A) was mutated by replacing part of its exon 3 and the complete exon 4 (32) with a neomycin-resistance gene (neo<sup>r</sup>). Breeding of heterozygous mice generated β3 homozygous (β3−/−) mice at the rate expected from the Mendelian frequency (160 +/+, 307 +/- and 144 −/−). The β3−/− mice grew normally, lived longer than one year, were fertile and had no obvious symptoms. Offsprings were genotyped for the β3 mutation by Southern blot analysis. Wild-type and mutant alleles were indicated by the presence of a 12 kb and a 8 kb EcoRI fragment, respectively (Fig. 1B). The deletion of the β3 gene was confirmed by Northern blot analysis (Fig. 1C) and immunoblotting of brain extracts (Fig. 1D). Additionally, we found no significant differences between wild-type and β3−/− mice in the expression levels of β1, β2 and β4 proteins in whole brain (Fig. 1D), suggesting that there was no apparent compensatory increase of the expression of other β subunits. Furthermore, it is likely that β3 subunits are also expressed in other parts of the nervous system. The dorsal root ganglions (DRGs) contain cell bodies of primary afferent sensory fibres, including myelinated A fibres and unmyelinated C fibres, which project into the spinal cord. Thus, DRG neurones represent a central component of pain pathways and, additionally, isolated DRG neurones represent a well known cell system in studies of neuronal calcium channels (31). Since the expression pattern of β subunits in DRG neurones is not known, we analysed extracts of isolated DRGs using specific antibodies. Western blot analysis detected the β3 protein in DRGs from control animals but not from β3−/− mice (Fig. 1E). These results indicate that the gene targeting strategy used in the present study results in suppression of β3 protein expression in the DRGs of the adult animals that were selected for further experiments. The protein level of the β1, β2 and β4 subunits was very low but detectable (Fig. 1E). As in brain extracts, no significant change in the expression level of β1, β2 and β4 was apparent in extracts of DRGs isolated from β3−/− mice.

Nociception is altered in β3−/− mice

Since ablation of β3 suppresses the expression of β3 subunits in the brain and sensory neurones (Fig. 1), it is likely that the deletion of the β3 subunit might have impact on neuronal functioning in various regions of the nervous system and, thus, at different levels of pain processing pathways. It is known that blockers of N-type calcium channels exert spinal antinociceptive actions (37-39). Additionally, P/Q-type calcium channels may be also
involved in nociceptive processes at the spinal level (40, 38). Since β3 subunits associate to form N- and P/Q-type calcium channels (21, 22, 41), the suppression of β3 subunit expression (Fig. 1) produces likely major defects in the nociceptive pathway and, therefore, we analysed the pain related phenotype of the β3-/- mice in behavioural test for thermal, mechanical and chemical nociception (42). Responses to acute thermal stimuli were studied using the tail flick (Fig. 2A) and hot plate tests (Fig. 2B). Tail flick is primarily a spinal reflex, whereas a substantial supraspinal component, which involves lifting and licking of the hind paw, is required in the hot plate assay (35). Tail flick latencies were longer in the β3-/- mice both when the tail was exposed to heat by irradiation and by immersion in water at 52 °C (Fig. 2A). In the hot plate test, the latencies for licking were also increased and the delay for escape jumping was almost doubled in the β3-/- mice (Fig. 2B). Thus, independent of whether spinal or supraspinal components are engaged in the nociceptive response, the thresholds for thermal stimuli appear to be higher in the β3-/- mice. By contrast, the responses to acute mechanical stimuli appear to be normal in the β3-/- mice since the mechanical thresholds measured with von Frey filaments were similar to the thresholds of wild type mice (Fig. 2C; wild type, 8.12 ± 1.6 mN, n = 10; β3-/-, 6.15 ± 0.7 mN, n = 10). In order to study the role of the β3 subunit in persistent pain, we examine the behaviour of β3-/- mice employing CFA (complete Freund's adjuvant) and formalin. CFA was injected in the foot pad of the left hind paw and, within 24 - 60 h, induced swelling in the injected paw in wild type mice (injected paw, 2.35 ± 0.07 mm, contralateral side, 2.02 ± 0.07 mm, P < 0.05, n = 10) and β3-/- mice (injected paw, 2.47 ± 0.09 mm, contralateral side, 2.17 ± 0.06 mm, P < 0.05, n = 10). The degree of swelling of the injected paw was similar in both groups of mice. After 60 h, the mechanical thresholds measured on the ipsilateral injected paw (Fig. 2C; wild type, 1.65 ± 0.4 mN, n = 10; β3-/-, 1.40 ± 0.4 mN, n = 10) and on the contralateral paw (wild type, 5.21 ± 0.5 mN, n = 10; β3-/-, 3.87 ± 0.6 mN, n = 10) were significantly reduced (P < 0.05) in wild type and β3-/- mice when comparing them to the respective thresholds measured before the CFA injection (Fig. 2C; wild type, 8.12 ± 1.6 mN, n = 10; β3-/-, 6.15 ± 0.7 mN, n = 10). Thus, CFA induced hyperalgesia on the ipsilateral and contralateral side of injection in both groups of animals. However, no difference between wild type and β3-/- mice was detected in the mechanical thresholds measured after CFA induced swelling. It appears, therefore, that β3 subunits are not critical for the development of hyperalgesia in the CFA induced inflammatory pain. The neurochemical signature of the CFA induced pain is the internalisation of substance P receptors in laminae I, II - IV of the
spinal cord (43). Considering that N-type calcium channels are primarily restricted to laminae I - II (24, 25) and P type channels are localised in deeper laminae (30), it is likely that alterations of N-type channels in the β3-/- mice become more evident in specific assays of nociception, like the formalin induced inflammation, which is associated with internalisation of substance P receptors in lamina I (43). Therefore, we examined the responses of the β3-/- mice to chemical stimuli in the formalin test, which is also a well established model to study central sensitisation events at the spinal level after peripheral inflammatory states (35). Subcutaneous injection of formalin in the hind paw elicits biphasic pain responses in wild type and β3-/- mice (Fig. 3A). In the phase 1, formalin stimulates the nociceptors generating acute pain and, in phase 2, the inflammation induced by formalin elicits persistent pain. Both, wild type and β3-/- mice behaved similarly in phase 1 but the nociceptive behaviour during phase 2 was attenuated by 43% in β3-/- mice (Fig. 3A and B). Because the tests for thermal, chemical and mechanical nociception measure motor responses as endpoint, we examine possible motor/sedative defects in the β3-/- mice. In the rotorod test, the performance of wild type and β3-/- mice were indistinguishable (time on the rod: wild type, 64.62 ± 17.0 s, n = 8; β3-/-, 41.86 ± 9.8, n = 8), indicating that locomotor functions are not altered in the β3-/- mice and, thus, the behavioural tests used in the present study delineate the profile of nociception in the β3-/- mice. In summary, the β3-/- mice showed higher thresholds for acute thermal stimuli but the responses to mechanical stimuli were not altered either before nor after induction of inflammatory pain with CFA. The acute pain induced by formalin was not altered but the persistent pain observed in phase 2 of the formalin test was markedly reduced in the β3-/- mice.

**Neuronal activity in the spinal cord of β3 deficient mice**

Myelinated A fibres and small diameter, unmyelinated C fibres project to the spinal cord and form neuronal pathways that transmit nociceptive information from the peripheral site of injury to the brain. Among the multiple nociceptive pathways, the spinothalamic pathway originates primarily from neurones in the neck of the dorsal horn and terminates in the ventroposterior and ventrobasal thalamus (44). Within the spinal cord, L-type calcium channels are present in the dorsal horn with a density comparable to brain areas. N-type calcium channels show the highest densities in the superficial laminae of the spinal cord (24, 25, 30) and P-type calcium channels in the deeper laminae (30). Since the response to inflammatory pain appears to be preferentially damped by the deletion of β3 subunits, we
examine in vivo neuronal responses in the spinal cord evoked by stimulation of peripheral nerves. The neuronal activity was recorded deep in the spinal segment Th8, where responsive units of the spinothalamic pathway are expected, and the stimuli were delivered to the sural nerve of the contralateral side (see Methods). Under these conditions, trains of pulses with amplitudes up to 30 V, which were applied at a frequency of 0.2 pulses per second (PPS), evoked a volley of spikes that lasted less than 100 ms (Fig. 4A). With pulse amplitudes of at least 50 V, we detected in wild type and β3-/− mice at least two types of responsive units that can be distinguished by the latencies of the evoked spikes (Fig. 4A, lower panels). The latencies shorter than 70 ms probably reflects the contribution of A fibres while longer latencies likely reflect the activation of C fibres. In order to compare the thresholds for the activation of responsive units in wild type and β3-/− mice, we analysed the spinal activity evoked by pulses with amplitudes between 10 V and 100 V. Under the present experimental conditions, stimuli with amplitudes above 50 V evoked maximal spinal activity in wild type and β3-/− mice (Fig. 4B). The activity evoked in the spinal cord, however, depends on the frequency of stimulation. During trains of high frequency pulses, the neuronal activity increases spontaneously, a phenomenon that is observed in various nociceptive pathways and is known as wind-up (45). Thus, in order to examine the wind-up, we selected an amplitude of 50 V to evoke maximal responses (Fig. 4B) and applied trains of pulses with this amplitude at frequencies higher than 0.2 PPS. In several experiments with wild type mice, a moderate wind-up with a delay of 10 - 15 s was observed with a frequency of 0.5 PPS (not shown). Therefore, we compared the wind up in wild type and β3-/− mice at a frequency of 1 PPS (Fig. 5). In experiments with wild type mice, we consistently observed maximal activity after 5 - 7 s of repetitive stimulation, which were accounted by the increase of spikes with latencies longer than 100 ms (Fig. 5A). Similar wind-up of the spinal cord activity was not observed in paired experiments with β3-/− mice (Fig. 5A and B). Thus, this first characterisation of the spinal cord activity in vivo suggests that the activation thresholds of responsive units appears to be not affected but the wind-up is absent in the β3-/− mice.

**Calcium channel currents in DRG neurones**

The previous results pinpoint the nociceptive defects resulting from the deletion of β3 subunits to the spinal level (Fig. 2-5). Since the expression of β1, β2 and β4 subunits appears unaltered in DRG neurones of β3 deficient mice (Fig. 1), the α1A, α1B, α1C, α1D and α1E subunits, which have been detected in murine DRG neurones (46) might be associated either with β1, β2 or β4 subunits in the β3 deficient mice. In order to determine the functioning of neuronal
calcium channels in the β3 deficient mice, we recorded whole cell barium currents (I_{Ba}) in isolated DRG neurones. Depolarising voltage steps were applied from holding potentials of -50 and -110 mV and difference currents were obtained by subtracting the records at -100 mV from the respective traces at -50 mV. (Fig. 6). The large variability of DRG neurones concerning cell size and calcium channel expression (47, 48) precluded, however, a quantitative analysis of current amplitudes. Another observation in this series of experiments was that the activation and inactivation time courses of compound and difference currents were similar in β3-/- DRG neurones and controls (Fig. 6A). Since the expression of β subunits speeds up the activation and inactivation of recombinant calcium channels (e.g., 10) and antisense depletion of β subunits produces the converse effects in DRG neurones (49), this observation indicates that β1, β2 or β4 are able to replace β3, at least, in what concerns activation and inactivation.

Additionally, the coexpression of β with α1 subunits shifts the membrane potential required for channel opening to more hyperpolarised potentials (1, 2). We observed that the peak of the aggregate whole cell current measured at a holding potential of -110 mV was typically at -10 mV in control and at 0 mV in the β3-/- mutants (Fig. 6A). As can be seen in the normalised current-voltage relationships obtained at a holding potential of -70 mV (Fig. 6B), the whole cell current activation is shifted by ~8 mV to more depolarised potential in β3-/- neurones when compared to wild type. The activation midpoints in the normalised curves of Fig. 6A are -15.4 mV and -23.5 mV for β3-/- and wild type neurones, respectively. When the current voltage relationships of the individual neurones were fitted with a Boltzmann equation as described previously (50), the calculated potentials for half activation in β3-/- and wild type neurones were -9.1 ± 1.1 mV (n = 17) and -18.1 ± 2.1 mV (n = 11), respectively. A similar shift in the voltage dependence of calcium channel currents was observed in sensory neurones treated with generic anti-β-antisense oligonucleotides (50). Since β subunits have been implicated in the G protein mediated inhibition and voltage dependent facilitation of neuronal calcium currents (13, 14), an abnormal modulation of whole cell calcium channel current by G proteins is expected in the β3 deficient mice. As previously reported (51, 52), we found that activation of G proteins with GTP-γ-S slowed the activation kinetics of calcium channel currents and reduced the current amplitude in wild type DRG neurones (Fig. 7A). Depolarising prepulses accelerated the activation of whole cell calcium channel currents, increased the current amplitude and shifted the current-voltage relationship to more negative potentials (Fig. 7A), in line with the voltage dependent unblocking of G protein mediated inhibition of neuronal calcium channels (53, 54). The prepulse facilitation resulted in a voltage dependent facilitation of calcium channel currents.
By contrast, we found that depolarising prepulses speed up the activation time course of whole cell calcium channel currents after dialysis of GTP-γ-S in the majority of \( \beta^3-/- \) DRG neurones (9 out of 13), but no increase of current amplitude was observed and, accordingly, there was no shift in the current-voltage relationship (Fig. 7B). Thus, the main difference between this group of \( \beta^3-/- \) neurones and wild type neurones is observed in the degree of prepulse facilitation of whole cell calcium currents (Fig. 8A and B). This observation is in line with previous studies, which showed that the degree of facilitation of recombinant calcium channels is strongly dependent on the expressed \( \beta \) subunit, although the activation time course of whole cell currents facilitated by a prepulse is similar for all \( \beta \) subunits (55). In the remaining \( \beta^3-/- \) DRG neurones (4 out of 13), GTP-γ-S had no effect at all and depolarising prepulses produced a strong inactivation of calcium channel currents (Fig. 8B). All in all, this first electrophysiological characterisation of DRG neurones of \( \beta^3 \) deficient mice indicates that the voltage dependent facilitation of calcium channel currents is altered, the voltage dependence of the activation is shifted to more depolarised potentials, although the time course of activation and inactivation is apparently normal.

Expression of calcium channel \( \alpha_{1B} \) subunits in dorsal root ganglions

In murine DRG neurones, \( \alpha_{1B}, \alpha_{1A} \) as well as \( \alpha_{1C}, \alpha_{1D} \) and \( \alpha_{1E} \) have been detected with specific antibodies (46). Considering this wide expression of \( \alpha_1 \) subunits in DRG neurones, the deletion of the \( \beta^3 \) subunit (Fig. 1) could alter in principle N-, P/Q-, L- and R-type calcium channels. In sympathetic neurones of \( \beta^3 \) deficient mice, N-type calcium channel current densities are reduced whilst P/Q-type current densities appear to be normal (19). At the synaptic sites in the spinal cord, N-type calcium channels appear to mediate neurotransmitter release (56, 57). As a consequence, \( \beta^3 \) deletion could be important for nociception and sensory processing due to the reduction of N-type calcium channels in DRG neurones, as indicated by our whole-cell current recordings (Fig. 6). Since the N-type component of calcium currents appears to be variable within the heterogeneous population of DRG neurones (47, 48), we first analysed the expression of \( \alpha_{1B} \) in DRG homogenates using binding assays with \( ^{125}\text{I}-\omega\)-conotoxin GVIA, a N-type channel blocker. The specific binding estimated with increasing concentrations of \( ^{125}\text{I}-\omega\)-conotoxin GVIA was reduced by a factor of ~2 in homogenates of \( \beta^3-/- \) DRG neurones (Fig. 9A). This result suggests that the expression of \( \alpha_{1B} \) is reduced in the mutant. Since \( \beta \) subunits control the membrane targeting of \( \alpha_1 \) subunits, we repeated binding assays with intact neurones in order to estimate the expression of \( \alpha_{1B} \) in the membrane.
Similarly as in the experiments with DRG homogenates (Fig. 9A), the specific binding at a saturating concentration of the N-type blocker was reduced in intact DRG neurones (Fig. 9B), indicating that the number of N-type calcium channels present in the membrane of DRG neurones is reduced in the β3 deficient mice. Since these observations suggest that the proportion of N-type currents is reduced in the mutant mice, we determine the inhibitory effects of ω-conotoxin GVIA on whole cell currents of DRG neurones. As reported previously for sympathetic neurones (19), we observed that the percentage of current inhibition by ω-conotoxin GVIA ranges from 37 % to 70 % in wild type and from 0 % to 68 % in β3-/- DRG neurones. The cumulative distributions indicate, however, that the percentage of inhibition is often less than 25 % in β3-/- DRG neurones (Fig. 9C, lower panel). In average, the inhibitory effects of ω-conotoxin GVIA are less pronounced in β3-/- than in wild type DRG neurones (Fig. 9C, upper panel). Thus our binding studies and pharmacological experiments with ω-conotoxin GVIA imply that the number of functional N-type calcium channels and, consequently, the proportion of N-type calcium channel currents are reduced in DRG neurones of β3 deficient mice.
DISCUSSION

In the present study, we examined the role of the β3 subunit of calcium channels in the pain perception. The use of a gene targeting strategy to suppress the expression of β3 subunits allowed the analysis throughout various levels, including protein expression and calcium channel currents in single DRG neurones, functioning of neuronal circuits in the spinal cord of anaesthetised animals and nociceptive behaviour in awake mice. Our results show that β3 subunits are critically involved in the function of nociceptive pathways and, thus, delineate the in vivo function of one of the so called auxiliary subunits of voltage dependent calcium channels.

In behavioural assays, we studied the thermal, mechanical and chemical nociception of the β3-/- mice. Responses to acute mechanical and thermal stimuli were not altered or only moderately increased in the β3-/- mice, respectively (Fig. 2). Similarly, the thresholds for activation of responsive units in the spinothalamic pathway were not apparently changed in the β3-/- mice (Fig. 4). However, the formalin test, which is based on the measurement of licking/biting time as a robust predictor for nociception (42), revealed that β3-/- mice develop weak hyperalgesia/allodynia after inflammation because the nociceptive behaviour was strongly damped in the tonic, inflammatory phase 2 (Fig. 3). In this respect, the response of β3-/- mice compares to the behaviour of mice lacking the α1B subunit, which also develop weak hyperalgesia/allodynia after formalin inflammation (26-28). This parallel is in accordance with our finding that DRG neurones of β3-/- mice have less binding sites for ω-conotoxin GVIA, a blocker of N-type calcium channels (Fig. 9). Our binding and pharmacological studies with ω-conotoxin GVIA (Fig. 9), furthermore, also suggest that the deletion of the β3 subunit reduced the membrane expression of α1B. The contribution of L-type calcium channel currents to the development of formalin induced hyperalgesia seems unlikely, although our results do not rule out a reduction of L-type current in DRG neurones of β3-/- mice. When L-type blockers are administered systemically, antinociceptive effects have been observed in rats using the formalin test (e.g., 58). However, direct application of L-type blockers to the surface of the spinal cord, which is equivalent to intrathecal injection, has no effect on the excitability of dorsal horn neurones produced by formalin inflammation in the rat (38). Accordingly, intrathecal injection of specific blockers of L-type calcium channels has no effect on the nociceptive behaviour of rodents either during the early phase
nor during the late phase of the formalin test (29, 39). Unfortunately, a comparison between the phenotype of β3, α1C and α1A deficient mice is not possible because α1C (Cav1.2) and α1A (Cav2.1) deletion were embryonic lethal and generate strong neurological deficits, respectively (59, 60). Nevertheless, application of the P-type blocker ω-agatoxin IVA to the surface of the spinal cord selectively blocks the excitability of dorsal horn neurones in phase 2 (38) and, accordingly, intrathecal injection of the P-type blocker reduces the nociceptive behaviour during the late phase but not during the acute phase of the formalin test in the rat (40). On the other hand, intrathecal injection of the N-type blocker ω-conotoxin GVIA reduces the nociceptive behaviour in phase 1 and 2 of the formalin test in mice (39) and rats (29). In the formalin tests that were repeated independently in three laboratories of the authors, no clear differences were observed in the behaviour of wild type and β3/-/ mice during the early phase (Fig. 9). Mice deficient in α1B subunits also behaved normally in the phase 1 (26-28). Although the unaltered phase 1 in α1B and β3 deficient mice may reflect compensatory mechanisms, the possibility remains that defects of P-type calcium channels may account partially for the reduced nociceptive responses of β3/-/ mice in the formalin test. We compared the pain related behaviour of the β3/-/ mice in formalin and CFA tests, which are based on inflammatory processes that produce distinct neurochemical signatures in the spinal cord. The CFA induced inflammation produces internalisation of substance P receptors in laminae I, II - IV of the spinal cord, while the formalin induced inflammation appears to induce substance P receptor internalisation in lamina I (43). Considering that N-type calcium channels are primarily restricted to laminae I - II (24, 25) and P type channels are localised in deeper laminae (30), our results (Fig. 2 and 3) favour the in vivo importance of a possible modification of spinal N-type channels by deletion of β3 subunits and underscore the importance of possible alterations of P-type calcium channels. Furthermore, deletion of β3 subunits appears to have not much effect on the expression but alters the voltage dependent activation of P/Q type calcium channels, at least in sympathetic neurones (19). In the bodies of β3/-/ DRG neurones, we observed a depolarising shift in the voltage dependence of the activation of compound calcium channels currents (Fig. 6). Thus, deletion of β3 alters the function of calcium channels that are expressed in the membrane, in way that makes them less sensitive to membrane depolarisation. Additionally, the voltage dependent facilitation of compound calcium channel currents (Fig. 7 and 8) and, likely, the G protein modulation of calcium channels are altered in β3/-/ DRG neurones. These defects in the activation and G protein modulation of the expressed calcium channels as well as the low expression of N-type
calcium channels (Fig. 9) likely modify the pain perception at the spinal level. In order to support this conclusion, we studied responsive units in the spinothalamic pathway. In wild type but not in β3-/- mice, high frequency stimulation of the sural nerve induced wind-up, which probably represents sensitisation at the spinal level (Fig. 5). This defect in the processing of pain perception at the spinal level likely reflects the reduced expression of N-type calcium channels together with altered calcium channel currents induced by the ablation of β3. By contrast, spinal and supraspinal nociceptive components account for the low sensitivity in the formalin test of mice lacking the α1E subunit (Ca2.3; 61).

The importance of calcium channel auxiliary subunits in vivo is underlined by the role of α2δ and β subunits in neuroplasticity after nerve injury (62) and in pathological states such as cardiac disfunction (15) and diabetes (16), respectively. The results of the present study suggest an important in vivo function of β3 subunits in the nervous system, specifically, in the pain processing at the level of spinal cord. It is not known whether the expression of β3 subunits is affected in pathological states but the specific nociceptive action of the deletion of β3 subunits indicates that, like α1B (63) and α2δ (64), the β3 subunits are important pharmacological targets for the modulation of pain in pathological states.

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FIGURE LEGENDS

**Figure 1.** Targeted disruption of the calcium channel β3 gene. A. β3 locus. Organization of exons (*filled boxes*) and introns (*lines*). Targeting vector IIMB, second diagram: A 1kb HincII (H) fragment, containing part of exon 3, exon 4 and part of the following intron was replaced by the neomycin resistance cassette (*neo*). The bottom diagram shows the structure of the homologous recombination product. Abbreviations: E, EcoRI; P, probe; tk, herpes simplex virus thymidin kinase gene. B. Identification of β3+/+, β3+/- and β3-/- mice by Southern (DNA) blot analysis. C. Northern blot of total RNA extracted from whole brain of β3+/+, β3+/- and β3-/- mice. The blot was hybridized with mouse β3 cDNA sequences ( nucleotides 291 to 602 ); lower panel, hybridization of the same filter with human β-actin cDNA. D,E. Immunoblot analysis of β3 (top), β1, β2 and β4 (bottom) expression in brain (D) an in DRGs (E).

**Figure 2.** Nociceptive responses of wild type (+/+) and β3 deficient mice (-/-) to thermal and mechanical stimuli. A. Tail flick latency to noxius heat (heat irradiation, n = 13 for both groups; tail immersion at 52°C, n = 14 for both groups). B. Hot plate test at 56°C (n = 14 for both groups). C. Response to mechanical stimulation with von Frey hairs before and 60 h after injection of CFA (-CFA and +CFA, respectively; n = 10 for both groups). All data are mean ± S.E.M. and were analysed by the Mann-Whitney U-test (*, P<0.05).

**Figure 3.** Nociceptive behaviour of wild type (+/+ ) and β3 deficient mice (-/- ) after subcutaneous injection of formalin into the hind paw. A. Time course of licking and biting behaviour. B. Total duration of nociceptive responses during phase 1 and 2. Data are mean ± S.E.M. ( n = 7 for both groups ) and were analysed by the Mann-Whitney U-test (*, P<0.05).

**Figure 4.** Neuronal activity in the spinal cord. Voltage pulses with amplitudes between 10 V and 100 V were delivered to the sural nerve at a frequency of 0.2 pulses per second (PPS) and the evoked activity was recorded in the contralateral spinal segment Th8. A. Representative recordings of spikes (*upper panels*) produced by responsive units in experiments with a wild type (+/+ ) and a β3-/- mouse (-/- ). Voltage pulses with the indicated amplitudes were applied at the time points indicated by arrows. The histograms of spike latencies (*lower panels*) were compiled from the responses to trains of 12 pulses with an amplitude of 50 V. B. Thresholds of responsive units. The spikes observed within 300 ms
after each voltage pulse were counted and averaged for trains of 12 pulses (frequency, 0.2 PPS; duration, 5 ms). To estimate the activity evoked by a given pulse amplitude, the averaged number of spikes of the corresponding train of pulses was normalised to the maximal value obtained with pulse amplitudes between 10V and 100 V. Each symbol represents a different experiment (wild type mice, upper panel, n = 5; β3/- mice, lower panel, n = 4). Stimuli above 50 V produced maximal spinal cord activity in wild type and β3/- mice.

Figure 5. Spinal cord activity during high frequency stimulation. Pulses of 50 V amplitude that evoked maximal spinal cord activity (Fig. 4B) were applied at a frequency of 1 pulse per second (PPS). A. High frequency stimulation of the responsive units characterised in Fig. 6A. During a train of 16 pulses (upper panel), the evoked activity increased rapidly within the first 5 s (wind-up) and remained elevated up to 16 s in the experiment with the wild type mouse (+/+, middle panel). Similar wind-up was not observed in the β3/- mouse (-/-, lower panel). B. Wind-up during high frequency (1 PPS) trains of pulses (amplitude, 50 V; duration, 5 ms). The increase of evoked activity was estimated as the number of spikes, which were observed within 500 ms after each pulse, divided by the number of spikes evoked by the first pulse. Different symbols represent the mice characterised in Fig. 6B (wild type mice, left panel, n = 5; β3/- mice, right panel, n = 4).

Figure 6. Voltage dependence of calcium channel currents in DRG neurones. Whole cell currents (I_Ba) were evoked from various holding potentials (HP) with depolarising pulses to potentials between -40 mV and +40 mV in 10 mV increments. A. Representative whole cell current families from DRG neurones of wild type (left panels, +/-) and β3/- mice (right panels, -/-) elicited from the indicated holding potentials. Difference currents were obtained by subtracting current traces at HP = -50 mV from the respective traces at HP = -110 mV. In the wild type neurone, the I-V relationships obtained with holding potentials of -110 mV (continuous line) and -50 mV (dotted line) show peaks of I_Ba amplitude at -10 mV and 0 mV, respectively. I_Ba evoked in the β3/- neurone from both holding potentials peaked at 0 mV. B. Activation of whole cell currents in response to depolarising pulses from a holding potential of -70 mV. Current amplitudes were normalised (filled circles, continuous line: wild type, n = 11; open circles, dotted line: β3/- neurones, n = 17). The activation midpoints were -23.5 mV and -15.4 mV for wild type and β3/- neurones, respectively.
Figure 7. Voltage dependent facilitation of calcium channel currents in DRG neurones. After cell dialysis with GTP-γ-S (500 µM), whole cell currents were elicited with test pulses from a holding potential of -70 mV to potentials between -30 mV and +30 mV. The voltage dependent facilitation was induced with a prespule to +80 mV. Superimposed are consecutive whole cell current traces obtained without (1) and with (2) prepuless at the test potentials indicated on the left (upper panels). The I-V relationships (bottom panels) show whole cell current amplitudes measured 10 ms after onset of test pulses without (1, dotted line) and with prepulse (2, continuous line). The representative example from a wild type DRG neurone (A) shows the slow activation of calcium channel currents after dialysis with GTP-γ-S (1) and the prepulse dependent facilitation (2). Prepuless induced also a shift of the I-V relationship to hyperpolarising potentials. As illustrated in the recordings from a β3-/- neurone (B), prepuless speeded up the activation of whole cell currents in β3-/- DRG neurones but had almost no effect on the I-V relationship.

Figure 8. Prepulse facilitation. Summary of experiments with DRG neurones in the absence (control) and presence of GTP-γ-S (500 µM). Calcium channel current amplitudes were measured 10 ms (full circles) and 49 ms (open squares) after the onset of the test pulse. Facilitation was calculated as the ratio of currents amplitudes obtained with and without the prepulse to +80 mV. A. Prepulse facilitation is seen in wild type neurones at all potentials (n = 12). B. The majority of β3-/- neurones exhibited almost no prepulse facilitation (middle panel, n = 9). In an small number of β3-/- neurones (right panel, n = 4), prepuless induced strong inactivation of whole cell currents (inset; 1, no prepulse; 2, with prepulse; calibration bars, 20 ms and 500 pA). The values are mean ± S.E.M.

Figure 9. Binding of ω-conotoxin GVIA (ωCTX) to DRG neurones. A. Specific ωCTX binding in homogenates of dorsal root ganglions from wild type (+/+, full circles) and β3-/- mice (-/-, open circles). Apparent Bmax values are significantly different (+/+: 25.08 fmol / mg protein (95% conf. interv. 0.76-1.27); -/-: 13.21 fmol / mg protein (95% conf. interv. 0.43-0.65)), whereas KD values do not differ significantly (KD-range 1.1 to 8.9 pM). Experiments have been performed in duplicate with DRGs isolated from 60 mice. B. Specific ωCTX binding to intact DRG neurones (~14,400 neurones per determination) isolated from wild type (filled box, n = 7) and β3-/- mice (open box, n = 6). C. Inhibition of whole-cell calcium channel currents by ωCTX (3µM) in DRG neurones. Whole cell currents were elicited by depolarisations (100 ms long) to -10 mV from a holding potential of -70 mV.
The percentage of inhibition was calculated from peak current amplitudes obtained before and after (50 - 100 s) bath application of \( \omega \text{CTX} \). Whole cell currents of \( \beta3/- \) neurones (upper panel, open box, \( n = 10 \)) are less susceptible to inhibition by \( \omega \text{CTX} \) than wild type neurones (upper panel, filled box, \( n = 6 \)). As seen in the cumulative probability plots for wild type (lower panel, continuous line, \( n = 6 \)) and \( \beta3/- \) neurones (lower panel, dotted line, \( n = 10 \)), \( \omega \text{CTX} \) rarely inhibits whole cell currents of \( \beta3/- \) neurones by more than 25%. The values represent mean \( \pm \) S.E.M., the asterisks indicate \( P < 0.05 \), Student’s t-test.
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Fig. 9

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Pain perception in mice lacking the β3 subunit of voltage-activated calcium channels

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