Early delivery and prolonged treatment with nimodipine prevents the development of spasticity after spinal cord injury in mice

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Spasticity, one of the most frequent comorbidities of spinal cord injury (SCI), disrupts motor recovery and quality of life. Despite major progress in neurorehabilitative and pharmacological approaches, therapeutic strategies for treating spasticity are lacking. Here, we show in a mouse model of chronic SCI that treatment with nimodipine—an L-type calcium channel blocker already approved from the European Medicine Agency and from the U.S. Food and Drug Administration—starting in the acute phase of SCI completely prevents the development of spasticity measured as increased muscle tone and spontaneous spasms. The aberrant muscle activities associated with spasticity remain inhibited even after termination of the treatment. Constitutive and conditional silencing of the L-type calcium channel CaV1.3 in neuronal subtypes demonstrated that this channel mediated the preventive effect of nimodipine on spasticity after SCI. This study identifies a treatment protocol and suggests that targeting CaV1.3 could prevent spasticity after SCI.

INTRODUCTION

Spinal cord injury (SCI) in humans is a traumatic life-changing event that results in permanent sensory-motor disabilities. Traumatic SCI is characterized by an acute mechanical injury, resulting in the depression of spinal reflexes and absence of movements (1, 2). The initial insult is followed by a series of secondary damage to the injury epicenter and initially spared distal regions (1). Several months after the lesion, a substantial number of people with SCI (70%) develop spasticity (3), a maladaptive state of hyperexcitability of the spinal circuitry resulting in the appearance of tonic muscle contractions and episodic muscle spasms (4, 5). Spasticity impairs residual motor function, negatively affects the quality of life, and even leads to early mortality (6). Therefore, spasticity is a considerable problem for people with SCI (3). Despite major progress in neurorehabilitative and therapeutic approaches, no curative treatments exist. Thus far, neurorehabilitation facilitates moderate motor recovery but is not effective in alleviating the hyperexcitability of the spinal circuitry (7, 8). In contrast, the main pharmacological strategies suppress not only the hyperexcitability but also any remaining motor responses (9, 10).

L-type calcium currents represent a major component of the persistent inward currents (PICs) underlying plateau potentials, a membrane property that allows neurons to sustain firing with little or no synaptic inputs (11–13). The expression of plateau potentials is enhanced in spinal neurons during the chronic phase of SCI with a direct correlation between expression of plateau potentials and appearance of spasticity (12, 14–16). In addition to a role in membrane excitability, L-type voltage-gated calcium channels are involved in calcium signaling and calcium-induced gene expression in neurons (17). Intracellular calcium concentration increases after SCI and remains high for days, activating downstream signaling networks that alter gene expression (18–21). Induction of these processes can lead to neuronal death, which can exacerbate the primary injury and result in long-lasting changes to network function (22, 23).

Because L-type calcium channels may be implicated in the appearing of spasticity after SCI, we evaluated the therapeutic potentials of nimodipine, an L-type calcium channel antagonist and U.S. Food and Drug Administration–approved compound. We demonstrate that nimodipine administration initiated early after SCI and, for prolonged time, is capable of preventing the development of spasticity in a mouse model of SCI. Using transgenic mice, we provide a detailed expression profile of CaV1.3 channels in different neuronal and nonneuronal elements of the spinal cord and show that constitutive knockout (KO) or deletion of CaV1.3 channels in neurons is sufficient to prevent the development of increased muscle tone and spasms in the chronic phase of SCI. The study suggests that nimodipine might be a potential therapeutic option for long-lasting treatment of spasticity in humans after SCI.

RESULTS

Nimodipine prevents the development of tonic muscle contraction and muscle spasms after SCI

To assess the role of L-type calcium channels in spasticity after SCI, we used a mouse model of SCI with a complete transection of the second sacral segment (S2) of the spinal cord that replicates clinical signs of human spasticity (15, 24–27). In this model of chronic SCI, lesioned wild-type (WT) mice soon after SCI exhibited a tail posture similar in shape to that seen in unlesioned mice (fig. S1A). However, an abnormal change in the tail posture gradually appeared, peaking 6 weeks after SCI (chronic state; fig. S1B) and remaining so thereafter (fig. S1) (15, 24, 25, 27, 28). Electromyography (EMG) recordings from tail muscles in lesioned WT mice revealed an increase in tonic muscle activity at the curvatures that well correlated to the appearance of the abnormal tail posture (unpaired t test, P < 0.001; fig. S1D). We described the extent of the tonic muscle contraction by a severity index, which uses tail bending angles and length as a proxy for changes in muscle tone (fig. S1, D to G; see also Materials and Methods). In addition to the tonic muscle contraction, spasticity was characterized

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by spontaneous recurrent episodic muscle spasms with large increases in muscle activity (fig. S1C) (15, 24). Small- and large-sized motor units were recruited simultaneously at the beginning of the spasms, inducing an abrupt and temporary increase in tail bending (fig. S1C). Conversely, spasm termination appeared as decreased motor unit activity with successive muscle relaxation (fig. S1C). Because the increase in muscle tone and muscle spasm duration provide quantitative measurements of spasticity and reflect clinical signs of spasticity after SCI, we use these two parameters to evaluate the degree of spasticity in our study.

To evaluate the therapeutic action of blocking L-type calcium channels after SCI and its translational potential in humans, we administered subcutaneously nimodipine (10 mg/kg). Nimodipine is an L-type calcium blocker used for treatment of cardiovascular diseases as well as a protective agent for preventing worsening of neurological disorders after stroke (29). The drug efficiently passes the blood-brain barrier, allowing systemic delivery with no major side effects (30–32). Three groups of lesioned mice were used for this study. A first group of WT lesioned mice received just vehicle solution subcutaneously once a day from day 1 after SCI and every day for 6 weeks (vehicle group; Fig. 1A). A second group received nimodipine once a day from day 1 after SCI and every day for 6 weeks (early treated). A third group received nimodipine once a day from week 6 after SCI when spasticity is fully developed and stable and every day for 6 weeks (late treated group; Fig. 1A). The severity index and the spasm duration for all groups were assessed on the last day of treatment. Vehicle-treated animals showed strong tail bending (0.77 ± 0.04) and sustained occurrence of spasms (0.53 ± 0.03). In stark contrast, the early treated group exhibited a marked reduction of the severity index (0.1 ± 0.02) and muscle spasms (0.1 ± 0.025) when compared to the vehicle treated group (Fig. 1, B and C). The late treated also showed a reduction of the severity index (0.36 ± 0.03) and muscle spasms (0.35 ± 0.05) when compared to the vehicle-treated animals (Fig. 1, B and C). However, this reduction was less pronounced than that observed in the early treated animals.

Together, the data indicate that early treatment with nimodipine after SCI efficiently reduces the tonic muscle contraction and muscle spasms occurring after SCI. The stronger effect of the early-onset treatment indicates that the early administration of nimodipine reinforces its action by additional effects on the neurons.

### Only early and prolonged delivery of nimodipine prevents development of tonic muscle contraction and muscle spasms after SCI in mice

To provide further evidence for additional effects induced by the early delivery of nimodipine, we took advantage of the possibility that the adult sacral spinal cord can be isolated 6 weeks after SCI when spasticity is fully developed (15, 24). In this way, we can study the spinal activity and the motor responses at cellular detail in animals that either received an early and prolonged nimodipine treatment or none at all. Because spinal excitatory glutamatergic interneurons play a pivotal role in triggering and maintaining spasms after SCI (15), we decided to visualize excitatory interneuronal activity in lesioned mice. For this, we used genetically driven calcium imaging in glutamatergic neurons, which express the vesicular glutamate transporter 2 (Vglut2), in the spinal cord. To obtain expression of calcium indicators in Vglut2-positive cells, we crossed Vglut2Cre with GCaMP3 mice. Motor neuron activity was recorded in ventral roots during simultaneous imaging of spinal interneuron activity during sensory-induced spasms [Fig. 2A, see (15)]. In spinal cords from lesioned nontreated animals, excitatory spinal interneurons activity was elicited by sensory stimulation, resulting in prolonged motor responses (black traces in Fig. 2B). Subsequent application of nimodipine resulted in a reduction of interneuronal activity (mean reduction, 45 ± 3.2%) and motor responses (main reduction, 53 ± 5.6%; gray traces in Fig. 2, B and C). This reduction induced by administration of nimodipine in vitro was similar to what was seen in vivo in the late treated animals (mean reduction in spasm duration compared to the vehicle group, 49 ± 5.2%). To understand the effect of early in vivo treatment, we analyzed the spinal cords from early and prolonged treated mice. Our results showed that the duration and the amplitude of the motor activity evoked by sensory stimulation were shorter than in the lesioned nontreated animals (mean reduction, 87 ± 11%; Fig. 2D). This result is also reminiscent of the spasm reduction observed in vivo in the early treated group (compared to the vehicle group, 85 ± 4.1% and 81 ± 2.3%, respectively). The similarity between the in vivo and in vitro results suggested that nimodipine may have a protective effect on the spinal circuitries only when given soon after SCI in mice that actually may persist after the end of the treatment.

To examine whether and to which extent nimodipine has a protective effect on development of spasticity, we measured tonic muscle contraction and muscle spasms in lesioned mice after the termination of the treatment in the early treated group. Ten days after the end of treatment, a time where the drug is presumably completely cleared from the body (Fig. 2, E to G) (31), there was a slight increase in spasm...
Genetic silencing of L-type calcium channels CaV1.3 abolishes tonic muscle contraction and muscle spasms after SCI

Next, we investigated which of the L-type calcium channels nimodipine is blocking to prevent spasticity. Because plateau potentials are triggered and driven by strong dendritic calcium current, we focused our study on the L-type calcium channels CaV1.3 that are mainly located in the dendritic branches of spinal neurons (17). However, L-type calcium channels cannot be pharmacologically distinguished (12, 16, 33, 34). We thus decided to use a genetic approach to answer the question. We therefore lesioned the sacral spinal cord of Cacna1d (calcium voltage-gated calcium channels CaV1.3) mice and treated them with vehicle or nimodipine. In contrast to the results in wild-type mice, the severity index reached the value of vehicle-treated animals (Fig. 2J). However, this long-lasting protective effect was present only when the treatment was delivered early and for prolonged time. In case of an early but shorter nimodipine treatment (from day 1 to 3 weeks after SCI), the muscle tone was reappearing 6 weeks after the end of the treatment (9 weeks after the SCI), where the severity index reached the value of vehicle-treated animals (Fig. 2J).

Fig. 2. Early and prolonged treatment with nimodipine prevents the development of tonic muscle contraction and muscle spasms after SCI. (A) Schematic of the sacral spinal cord preparation from a lesioned Vglut2Cre;GCaMP3 mouse kept in vitro with a ventral root recording electrode (black; MN) and a dorsal root stimulation electrode (red) attached as well as microscope for simultaneous imaging of the calcium dynamics of excitatory spinal interneurons. (B) Prolonged ventral root activity evoked by dorsal root stimulation (stimuli of 50 μs, 10 Hz, 15 μA) and calcium dynamics of an excitatory interneuron (ΔF/F) in a lesioned Vglut2Cre;GCaMP3 mouse 6 weeks after SCI before (black) and after bath application of nimodipine (gray). (C) Calcium responses for the spinal interneurons (INs; n = 16) and motor neurons (MNs; n = 9) in three lesioned mice after nimodipine application. Mean ± SEM. (D) Motor response duration recorded in the ventral root in spinal cords of vehicle (Ve; N = 8) and early prolonged treated (ET; N = 7) mice 6 weeks after SCI (mean ± SEM, Welch’s t test, ***P < 0.001). (E) Schematic of the time points (test) shown in (F) and (G). (F) Representative images of the tail of an early prolonged treated mouse the last day of treatment and 10 days after the end of the treatment. (G) Severity index (left, N = 10) and spasm duration (right, N = 9) in early prolonged treated mice at the last day of treatment (filled bar) and 10 days after the end of the treatment (open bar). Mean ± SEM, paired t test, *P < 0.05; n.s., P > 0.05. (H to J) Schematic of the experimental protocol (H), with representative images of the tail of an early prolonged treated (6 weeks) mouse (upper) and an early short treated (3 weeks) mouse (bottom) (I), and severity index for vehicle (N = 10, red), early short treated (N = 10, yellow), and early prolonged treated mice (N = 11, cyan) 6 weeks after the end of the treatment (J). Brown-Forsythe and Welch’s ANOVA tests followed by Dunnett’s multiple comparisons with individual variances computed for each comparison. (K to M) Schematic of the time point tested (K) and severity index 15 weeks after SCI (L) for vehicle (N = 10, red), late and prolonged (N = 10, blue), early and short (N = 10), and early and prolonged treated mice (N = 10, cyan). Brown-Forsythe and Welch’s ANOVA tests followed by Dunnett’s multiple comparisons with individual variances computed for each comparison. (M) Severity index for all pharmacologically treated mice over the entire experimental protocol (15 weeks). The measured time points (dots), the variation of the mean (dashed lines), and SEM (paler filled line) between time points in vehicle (red, N = 10), late prolonged treated (blue, N = 10), early shortly treated (yellow, N = 10), and early prolonged treated mice (cyan, N = 10).
channel subunit alpha1D) KO mice that exhibits a constitutive functional silencing of the CaV1.3 calcium channels (35) (CaV1.3 KO; fig. S2, A to C). In contrast, the tail of chronically lesioned CaV1.3 KO mice did not show chronic bending, with the gross tail appearance remarkably similar to unlesioned WT mice and early treated mice (Fig. 3B and fig. S3A). EMG recordings of the tail muscles showed little tonic activity and sporadic small amplitude spasms (Fig. 3B). In lesioned WT mice 6 weeks after the lesion, the severity index ranged from 0.65 to 0.95 (with a mean value of 0.05 ± 0.005; Fig. 3D and fig. S3, D and E), which was not significantly different from unlesioned WT mice (P > 0.05) but almost 10-fold lower than lesioned WT mice (fig. S3C). The severity index of the lesioned WT and CaV1.3 KO mice remained the same after the 12-week period of observation (Fig. 3G). Simultaneously, lesioned CaV1.3 KO mice exhibited a strong reduction in muscle spasm duration compared to lesioned WT mice (Fig. 3, E and F, and fig. S3B).

Together, these results demonstrate that silencing of CaV1.3 channels is sufficient to prevent spasticity after SCI. These results also suggest that the effects of the nimodipine treatment after SCI may be mainly mediated by the blockage of the CaV1.3 channels.

CaV1.3 calcium channels are abundantly expressed in neurons and nonneuronal elements of the spinal cord

Next, we examined the spatial distribution of CaV1.3 channel–expressing cells in the mouse spinal cord to identify which cell populations may be involved in the development of spasticity. For this, we used a CaV1.3 reporter allele that, upon Cre-mediated recombination, prevents the expression of the Cacna1d gene and simultaneously expresses enhanced green fluorescent protein (eGFP) from the CaV1.3 locus [Cacna1d-eGFPflex; fig. S2 and (36)]. We crossed HoxB8Cre (homeobox B8) mice (37) with the Cacna1d-eGFPflex reporter to obtain reporter expression in cells caudal to the fourth cervical segment (fig. S2C). CaV1.3 calcium channels were widely expressed in the spinal cord from early development (fig. S4, A to C) to adulthood (Fig. 4). About 55% (51 ± 2.1, mean ± SEM) of the spinal cells expressed CaV1.3 channels (CaV1.3+ cells; Fig. 4, A and B). CaV1.3+ cells were found along the rostrocaudal axis of the cord (Fig. 4, C and D) distributed across the dorsal (laminae I to V), intermediate (laminae VI, VII, and X), and ventral (laminae VIII and IX) laminae (Fig. 4, E to H). CaV1.3+ cells exhibited the highest density in the superficial dorsal horn in all segments (Fig. 4H) with a lower density along the dorsolateral and mediolateral axes of the cord (Fig. 4, E to H), following the typical neuronal distribution within the gray matter of the spinal cord (38).

To assess the expression of the CaV1.3 channels in different neural populations, we crossed the Cacna1d-eGFPflex with Vglut2Cre (39), VIAATCre (40) (vesicular inhibitory amino acid transporter), and ChATCre (41) (choline acetyltransferase) mice, leading to reporter expression in glutamatergic, GABAergic/glycinergic, and cholinergic neurons, respectively. By comparison with the HoxB8Cre, we found that most of the spinal CaV1.3+ cells were of neuronal origin, with 35% (±6.5) of them being Vglut2+, 25% (±5) being VIAAT+, and 5% (±2.1) being ChAT+ (Fig. 4, I and J). Almost all ChAT+ ventral horn motor neurons (somatic motor neurons) and lateral horn preganglionic motor neurons expressed CaV1.3 channels, whereas only a small proportion of cholinergic interneurons were CaV1.3+ cells (fig. S4, D to H). In conclusion, CaV1.3 channels are abundantly expressed in the spinal cord, including neuronal elements of each neurotransmitter-defined subtypes (65 ± 3.4%) and nonneuronal elements (35 ± 9.8%).

Neuronal CaV1.3 channels is directly involved in the generation of tonic muscle contraction and muscle spasms

Given the abundant expression of CaV1.3 channels in neuronal and nonneuronal elements of the spinal cord, we wondered whether the preventive effect of silencing the CaV1.3 channels on spasticity after SCI may be of neuronal origins. We have previously shown that both excitatory (Vglut2+) and inhibitory (VIAAT+) interneurons are recruited during spasms (15) and they are the largest spinal population expressing CaV1.3 channels (Fig. 4). We therefore generated Vglut2Cre; Cacna1d-eGFPflex/+/ (Vglut2−/−CaV1.3KO) and VIAATCre; Cacna1d-eGFPflex/flex (VIAATCaV1.3KO) mice that exhibited a silencing of the CaV1.3 channels in the excitatory and inhibitory neurons, respectively (Fig. 5A and fig. S5). To determine the specificity of the KO allele, we measured PICs in spinal neurons (42–44) from newborn mice with one functional copy of the channel (CaV1.3+/* or with the complete silencing of the CaV1.3 gene (CaV1.3−/−) in spinal neurons (fig. S5). The persistent sodium-dependent component was blocked by tetrodotoxin (TTX), leaving the persistent calcium-dependent and TTX-resistant PICs (42). We blocked L-type calcium currents with

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Fig. 3. Genetic silencing of L-type calcium channels CaV1.3 (Cacna1d) is sufficient to abolish tonic muscle contraction and muscle spasms after SCI. (A) Schematic of the experimental protocol. (B to D) Representative images (B) of tails from a lesioned WT mouse (red, left) and lesioned CaV1.3 KO mouse (gray, right) the day after (acute) and 6 weeks after (chronic) SCI. (C and D) EMG recordings from the ventral muscles of tail 6 weeks after SCI in a lesioned WT (C) and lesioned CaV1.3 KO mouse (D). (E and F) Severity index (E) and spasm duration (D) 6 weeks after SCI for lesioned WT (red, N = 11) and CaV1.3 KO mice (gray, KO, N = 8). Mean ± SEM, Welch’s t test, ***P < 0.001. (G) Severity index 12 weeks after SCI for WT (red, N = 11) and CaV1.3 KO mice (gray, N = 8). Dots indicate the measured time points, and variation of the mean and SEM are indicated by the dashed and filled lines, respectively. Multiple t tests. n.s., P > 0.05; ***P < 0.001.
Fig. 4. CaV1.3 channels are abundantly expressed in the mouse spinal cord. (A) Representative transverse sections (maximal projections) of the cervical, thoracic, lumbar, and sacral spinal cord in HoxB8Cre;Cacna1d-eGFPflex/+ adult mice. Scale bar, 200 μm. (B) Average number of cells expressing CaV1.3 channels (GFP, green) out of all spinal cells (Nissl) in the same section (n = 12, N = 3). (C) Digital coordinates of Cav1.3+ cells from the left spinal cords in (A). (D) Grand mean of CaV1.3+ cells in different segments of the spinal cord (data from (B)). (E) Laminar density distribution of Cav1.3+ cells with the highest density in yellow and lowest density in blue. (F to H) Digital normalization of the Cav1.3+ cell position (F) and subsequent clustering of Cav1.3+ positions in laminae (G and H) (cervical = 3657, thoracic = 3406, lumbar = 4114, sacral = 3029). (I) Transverse sections (maximal projections) of the sacral spinal cord from adult Vglut2Cre;Cacna1d-eGFPflex/+ VIAATCre;Cacna1d-eGFPflex/+ mice stained for eGFP (enhanced green fluorescent protein; green) and Nissl (blue), merged, and magnified (dotted square). Scale bar, 200 μm (20 μm in the magnified area). (J) Proportion of excitatory neurons (Vglut2+, N = 2, n = 8), inhibitory neurons (VIAAT+, N = 2, n = 8), motor neurons (ChAT+, N = 2, n = 8), and nonneuronal cells (others) expressing Cav1.3 channels (% of Nissl-stained cells).

nifedipine (another specific and common blocker for L-type calcium channels) (45). About half of the PICs (48.6 ± 4.5%) were generated by L-type calcium currents (dihydropyridine-sensitive) in Cav1.3KO, while the dihydropyridine-sensitive component in the Cav1.3KO was greatly reduced (28.1 ± 3.69%; fig. S5, A to C). The I–V curves of the dihydropyridine-sensitive current displayed similar activation and peak voltages, but they were markedly reduced in Cav1.3KO (fig. S5C). When measured by subtraction, Cav1.3 calcium component represents about 40% of the PICs in spinal neurons (fig. S5D). Together, these results show that Cav1.3KO allele efficiently silenced the Cav1.3 calcium channels in neurons and that a minor dihydropyridine-sensitive PIC, presumably generated by Cav1.2 calcium channels, was left in the Cav1.3KO mice.

Six weeks after complete transection injury, lesioned Vglut2Cav1.3KO and VIAATCav1.3KO mice exhibited a diminished bending of the tail at rest and lower muscle activity (Fig. 5, A to C). Both the tonic muscle contraction and muscle spasm duration were reduced in both the Vglut2Cav1.3KO and VIAATCav1.3KO mice compared to lesioned WT mice (Fig. 5D). However, the reduction of the severity index and the muscle spasm duration in these mice was less pronounced than in the full Cav1.3 KO (Fig. 5E). It is worth noting that the silencing of the channels in the excitatory neurons may additionally silence the Cav1.3 channels expressed in some Vglut2+ motor neurons (46–48).

These data demonstrate that the reduction in the tonic muscle contraction and muscle spasms after SCI in the Cav1.3 KO is linked to neuronal silencing of Cav1.3 channels. However, the more pronounced effect in the Cav1.3 KO (Fig. 3) likely reflects the role of multiple subpopulations of spinal neurons in the generation and maintenance of spasticity after SCI (15) with a possible contribution of Cav1.3 channels in nonneuronal cells (49, 50).

**DISCUSSION**

Spasticity, defined as muscle stiffness and muscle spasms, is highly associated with severe motor incomplete cervicothoracic injuries (3, 51, 52). The present study provides a basis for a therapeutic treatment of problematic spasticity after SCI. We demonstrate that nimodipine prevents the development of the aberrant motor activity with long-lasting effects on the motor symptoms in a mouse model of SCI. Its effect is optimal when given early after the SCI, and it is seemingly mediated by blocking the activity of the neuronal Cav1.3 channels. Together, these findings identify a mechanism for the development of spasticity and provide a potential therapeutic strategy for the prevention of spasticity after SCI in humans.

Baclofen represents the main antispastic medication for patients and heavily affects voluntary motor responses as well as cognitive functions (53). Nimodipine is a well-tolerated drug for management of cardiovascular diseases and subarachnoid stroke with no major side effects (29). It is currently under investigation for treatment of neurological diseases including Parkinson’s and multiple sclerosis.
in which cell vulnerability has been largely attributable to the increased cell excitability mediated by calcium influx through Cav1.3 calcium channels (49, 54, 55). Recently, a large observational cohort study described that early and only early application of gabapentinoids, negative modulators of calcium channels, lowering excitability of spinal neurons, and thus directly reduces muscle activity. L-type calcium channels are known to prolong neuronal firing in physiologic condition (11, 16, 60), and their currents in spinal neurons have been related to abnormal motor responses after peripheral and central injury (12, 16, 61). Larger calcium currents may be the result of changes in voltage-dependent activation and inactivation curves of the channels to more hyperpolarized potentials, as identified in humans presenting different neurological disorders with a common expression of spasticity (62–64) or altered upstream regulatory elements of the channels (28). Moreover, Cav1.3 calcium channels may increase neuronal excitability after injury indirectly by cooperative gating of clustered Cav1.3 channels (65), activating calcium activated nonspecific cation channels (16), or promoting activation of calmodulin or calcium-dependent calpains to increase sodium channel activity after SCI (66, 67).

The treatment protocols used here identified a specific therapeutic window that maximizes the drug effects and allows for a long-lasting protection. Because L-type calcium channels represent a major source of calcium ions in neurons and intracellular Ca^{2+} increases soon after trauma lasting for weeks after it (21), the early and prolonged treatment with nimodipine may simultaneously interfere with different calcium-dependent processes that contribute to reorganization of spinal circuits after SCI. First, genetic silencing of the Cav1.3 calcium channels indicates that nimodipine may directly act at the neuronal level. L-type calcium channels may induce influx of calcium ions that activate calcium-dependent lipases and/or proteases and inhibit mitochondrial function leading to cell death (68, 69), induced expression of genetic programs for axonal degeneration (70), and fiber sprouting and synaptic rearrangements (23, 57, 70, 71). In addition, Cav1.3 calcium channels and, thus, nimodipine block may affect neuronal function by reducing vasospasm (72), increasing remyelination of neuronal axons (73, 74), enhancing microglia apoptosis, and reducing astrocyte activation (49, 75), all processes that promote neuronal survival (76) and have been shown to be affected by nimodipine administration in the acute state of SCI (77, 78). Thus, the secondary effect of nimodipine may blunt secondary injury changes and enhance restorative processes, preventing maladaptive plasticity of spinal circuits typical of the chronic phase of SCI.

In conclusion, spasticity after SCI is characterized by multiple pathological events in different post-injury phases whose sequence and underlying cellular mechanisms are unknown. Here, we have identified Cav1.3 channels as triggers of multiple neural processes whose activity soon after injury leads to the development of spasticity after

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**Fig. 5. Involvement of the neuronal Cav1.3 channels in the appearance of tonic muscle contraction and muscle spasms after SCI.** (A and B) Images of mouse tails in Vglut2Cav1.3 KO mice with genetic silencing of the Cav1.3 calcium channels in excitatory (A, Vglut2Cav1.3KO) and in VIAATCre;Cacna1d-eGFP−/− mice with genetic silencing of the Cav1.3 calcium channels in inhibitory neurons (B, VIAATCav1.3KO) before and 6 weeks after SCI. (C) Representative EMG recordings from ventral muscles of the mouse tail in WT (C1), Cav1.3 KO (C2), Vglut2Cav1.3KO (C3), and VIAATCav1.3KO (C4) mice 6 weeks after SCI. (D) Quantification of severity index (right) and spasm duration (left) 6 weeks after SCI in WT (N = 11), Cav1.3 KO (N = 8), Vglut2Cav1.3KO (N = 5 in the left graph and N = 4 in the right graph), and VIAATCav1.3KO (N = 4) mice. Each dot indicates an animal. Mean ± SEM, Brown-Forsythe and Welch’s ANOVA tests followed by Dunnett’s multiple comparisons with individual variances computed for each comparison. *P < 0.05, **P < 0.01, ***P < 0.001.
The translational potential of the early and prolonged blockade of the channels with nimodipine may represent a major advance in treating spasticity after SCI.

**MATERIALS AND METHODS**

**Study design**

We investigated the impact of blocking the L-type calcium channels, with a specific focus on the Cav1.3 subtype, on the development of spasticity after SCI in vivo. All animal experiments and procedures were approved by the Norwegian Food and Veterinary Authority. The sample size was determined using a priori power analysis to obtain a minimum power of 0.8 with a minimum effect size of 15% (compared to the control group), the vehicle group for the pharmacological treatment, and the lesioned WT for the genetic silencing) using G*Power 3 (University of Dusseldorf). Mice were allocated in a random block design to different groups for surgeries and the in vivo experiments. The analyses were blind to the treatments or to the genotype. N values indicate the number of mice, whereas n represents technical replicates.

**Mice strains and primers**

The Vglut2-Cre mouse line was generated in the Kiehn laboratory (39) and has been used in multiple studies before (15, 79–81). The VIAAT-Cre mouse is a bacterial artificial chromosome (BAC) line (15, 40). The HoxB8-Cre mouse was generated by H. U. Zeilhofer (37), the α1d-deficient mice (Cav1.3 KO) is from the laboratory of J. Striessnig (35), and the CaacaId-eGFPflex is reported in (36). GCamp3flex/Cre flex mice were obtained from The Jackson Laboratory [stock no. 028764]. Mice of both sexes were used in this study. Specifically, females and males were allocated in the following experimental groups: vehicle group (5, 5), early and short group (9, 8), early and prolonged group (5, 5), late and prolonged group (9, 8), lesioned WT (6, 5), Cav1.3 KO (5, 5), and CaacaId-eGFPflex (3, 2), and VIAAT-Cav1.3KO (2, 2). Animals were housed in rooms at 22°C and 12:12 light-dark cycle.

For conditional deletion of the Cav1.3 gene from the glutamatergic (glutamnergic/GABAergic) population, Vglut2-Cre (VIAAT-Cre) mice were crossed with a homozygous CaacaId-eGFPflex mouse to obtain silencing of the second allele. Vglut2-Cav1.3KO mice (see fig. S1E) were then crossed back with another homozygous CaacaId-eGFPflex mouse line to obtain the silencing of the second allele (see also fig. S1).

Cav1.3 KO mice were genotyped with the following primers:

- CaV_Fw, 5′-GGAGTTGTATATCTTGTTAAGCC-3′; CaVKO_Re, 5′-CTCGTCTATATTCTAACTCCCTA-3′ (product sizes: 2448 base pairs (bp) from the recombined CaacaId locus and 1170 bp from the WT CaacaId locus). Primers for the CaacaId-eGFPflex were as follows:
- CaV_Re, 5′-GGAGTTGTATATCTTGTTAAGCC-3′; CaV_Fw, 5′-GGAGTTGTATATCTTGTTAAGCC-3′ (product sizes: 400 bp from the recombined CaacaId locus and 250 bp from the WT CaacaId locus).

**Surgery and postoperative care**

All surgical procedures were performed in adult mice (2 to 4 months old) as previously described (15, 24). Briefly, the animal was deeply anesthetized with isoflurane, and a vertical incision was made at the second lumbar vertebral body to expose the second sacral spinal segment. Xylocaine (1%) was applied locally, and the spinal cord tissue was aspirated using small glass pipette (diameter of 100 μm), paying attention not to damage the main arteries and veins around the cord. Once the lesion was completed, the muscles surrounding the spinal column and the skin were closed with one 6.0 silk suture to protect the exposed spinal column. The animal was single-caged, and a post-surgery treatment of buprenorphine (0.1 mg/kg) and carprofen (5 mg/kg) was given subcutaneously for 2 to 5 days. After about 7 days, the animal was caged again with its initial partner. Only animals with a complete lesion of the spinal cord visually inspected during the dissection were included in the study.

**Pharmacological treatment**

Nimodipine (Sigma) for subcutaneous injection was used as previously reported in the literature (10 mg/kg) (49, 82). Nimodipine was dissolved in the vehicle solution [2% dimethyl sulfoxide, 5% ethanol, 40% polyethylene glycol, and phosphate-buffered saline (PBS)] and kept in the dark. Nimodipine was subcutaneously injected once a day for up to 6 weeks of consecutive treatment. The vehicle solution without nimodipine was injected in a control group (vehicle) with the same protocol.

**Evaluation of tonic muscle contraction and muscle spasms after SCI**

The mice were restrained in a mouse restrainer with the tail hanging, free to move. Recording electrodes for EMG were inserted intramuscularly in the ventral part of the tail at the 7th–9th coccygeal vertebral body (rostral recordings) and at the 15th–17th coccygeal vertebral body (caudal recordings). The electrodes consisted of two twisted Teflon-coated platinum/iridium wires (diameter of 125 μm; WPI, code no. PTT0502). They were threaded through a 30 1/2-gauge hypodermic needle before the Teflon cover was removed from the tips, and the two electrodes were then inserted 1 to 1.5 mm apart in the tail. A single wire, prepared like the electrodes for the recordings, was used as ground electrode and inserted on the dorsal side of the 10th vertebral body. Because mice were spinalized, they did not feel any pain during this procedure. The proximal ends of the recording wires were connected to a differential amplifier (custom-made). The EMG signal was sampled with 5 KHz, band pass–filtered (60 to 1000 Hz), and digitalized (Digidata 1440A, Molecular Devices) for offline analysis using Spike2 (CED products).

The severity index used to describe the change in tail morphology after lesion was generated using pictures of tail of the restrained mice (see fig. S1E). The tail was divided into four points (a, b, c, and d) and thus three segments (A, B, and C). Three measurements were taken: (i) the angle between the segments A and B (θ1), (ii) the angle between the segments B and C (θ2), and (iii) the relationship between the length of all segments (length of the tail, A + B + C) and the shortest distance between the base of the tail (point a) and the tip of the tail (point d) (indicating a contraction of the tail, new segment D). Next, the angles were compared to their maximal value (120°) and normalized to the specific weight that was given to each parameter in the severity index. Because the first angle is the parameter that better captured the contraction of the tail, we gave it a larger weight (0.4) than the two other measurements (0.3 maximum value). Then, the parameters were summed to generate the severity as indicated by the following formula

\[
\text{Severity index } = (0.4 \times \frac{\theta_1}{120}) + (0.3 \times \frac{\theta_2}{120}) + (0.3 \times \frac{100 - D}{100})
\]

The greatest value (1) indicates the most severe tonic muscle contraction.
The duration of spasms was measured in a set recording time. The beginning of a spasm was determined when the firing frequency of the motor units increased above the baseline activity, and similarly, the spasms were considered terminated when the firing frequency returned to the baseline activity. In Results, spasm duration is normalized to the total recording time. By normalizing these data, we can compare values between animals and over time. This description captures the total effect of the pharmacological treatment and the genetic silencing, each of which may affect the different features of a spasm, from its triggering (frequency) to its maintenance (duration).

Ventral root recordings and calcium dynamics of excitatory neurons in the isolated spinal cord

The sacral spinal cord preparation was isolated as previously described (15, 24). Briefly, adult lesioned mice were anesthetized with isoflurane, and a laminectomy was performed from the 11th thoracic vertebral body. The spinal cord caudal to the second sacral segment was removed and placed in a chamber with oxygenated modified artificial cerebrospinal fluid (101 mM NaCl, 3.8 mM KCl, 4.8 mM MgCl₂, 1.3 mM MgSO₄, 1.2 mM KH₂PO₄, 10 mM HEPES, 1 mM CaCl₂, 25 mM glucose; oxygenated in 100% O₂). After removing the dura mater and shortening the roots, the spinal cord was placed in a perfusion chamber with normal Ringer solution (111 mM NaCl, 3 mM KCl, 11 mM glucose, 25 mM NaHCO₃, 1.25 mM MgSO₄, 1.1 mM KH₂PO₄, 2.5 mM CaCl₂, oxygenated in 95% O₂ and 5% CO₂). After recording, the sacral spinal cord was transversally cut at the coccygeal (Co1) segment and placed in the perfusion chamber. The dorsal and ventral horns facing the objective (40×). Illumination of the cord for excitation (470 to 490 nm) and visualization (520 to 560 nm) was obtained by a 100-W mercury lamp. Illumination was repeated three times for each condition with 1-min interval in between. Cells were excluded from the study if there were clear space clamp artifacts (for example, bumps and notches in the inward currents). Results are means ± SEM. Liquid junction potentials imposed by the whole-cell recordings were not corrected. Nimodipine or nifedipine (20 μM) was used to block L-type calcium currents in vitro (11).

Immunohistochemistry

Adult mice were anaesthetized with pentobarbital, perfused with 4% (w/v) paraformaldehyde (PFA) in PBS. Then, the spinal cord was dissected and postfixed for 2 hours in 4% PFA. Newborn mice (postnatal day 1 to 4) were decapitated, and the spinal cord was dissected and fixed for 2 hours like for the adult tissue. After the postfixation, the cords were rinsed in PBS, cryoprotected in 30% (w/v) sucrose in PBS overnight, and embedded in OCT mounting medium. Transverse sections (20 μm thick) were obtained on a cryostat. Sections were blocked in PBS supplemented with 5% (w/v) fetal bovine serum and 0.5% (w/v) Triton X-100 (blocking solution) before being incubated overnight in blocking solution at 4°C with one or several of the following primary antibodies: chicken anti-GFP (1:1000; Abcam) and goat anti-ChAT (1:100; Millipore). Secondary antibodies (Invitrogen) were incubated for 2 hours at room temperature. Fluorescent Nissl stain (NeuroTrace 647, Molecular Probes) was added during the secondary antibody incubation. Slides were rinsed, mounted in Vectashield medium, and scanned with a confocal microscope (Zeiss) using 10× and 20× objectives. Multiple channels were scanned sequentially to prevent fluorescence bleed.

Cell count

For experiments in both newborn and adult mice, 12 to 20 non-adjacent cryosections of the sacral spinal cord were counted. Neurons expressing the marker of interest were manually counted using a raw Z-stack confocal image and the cell counter plug-in in ImageJ (NIH). Simultaneously, five reference points were manually added to build a set of Cartesian axes, with the zero centered on the
central canal and the y axis parallel to the midline of the spinal cord and the x axis orthogonal to it. This way, all soma coordinates were translated and rotated to a common coordinate system. Two-dimensional Kernel density estimation was obtained using a MATLAB script (DataDensityPlot) and displayed as a contour plots, with the contour lines connecting points of equal density from 40 to 100% of the estimated density range with increment of 10%.

**Statistical analysis**

All statistical analyses were performed using Prism 8 (GraphPad software). When assumptions of normal distribution and homogeneity of variances were met, paired t tests and repeated-measures analysis of variance (ANOVA) were used. When data did not meet these assumptions, nonparametric tests were used.

Distribution of data was compared using a paired t test (Fig. 2G), Welch t test (Figs. 2D and 3, D and E, and fig. S1D), multiple t tests with significance correction using Holm-Sidak method (Fig. 3F), ordinary one-way ANOVA with Tukey’s post hoc test (Fig. 1C), Brown-Forsythe ANOVA with Dunnett’s T3 post hoc (Figs. 2, J to L, and S3 and fig. S3C), and repeated-measures two-way ANOVA with the Geisser-Greenhouse correction followed by Tukey’s post hoc with individual variances computed for each comparison (fig. S5D). The tests for the post hoc comparisons were determined by the distribution of the data and by consideration and possible interpretation of the scientific value of the results for the present study. For this purpose, Tukey’s multiple comparisons test was used when large effects were assessed and stronger statistical power was needed. In contrast, for data in which sample size and investigated effects were smaller, more stringent tests were used (for example, Sidak’s and Bonferroni’s post hoc). Statistical tests and power for each data set are summarized in table S1. All statistical tests were two-tailed. Most of the data are represented as scatterplot and power for each data set are summarized in table S1. All statistical analyses were performed using Prism 8 (GraphPad software).

**SUPPLEMENTARY MATERIALS**

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Fig. S1. Tonic muscle contraction and muscle spasms 6 weeks after SCI.

Fig. S2. Genetics of constitutive and the conditional KO Cacna1d mice used in the study.

Fig. S3. Tonic muscle contraction and spasms are abolished after SCI in mice with complete silencing of the Cav1.3 calcium channels.

Fig. S4. Expression of Cav1.3 channels in the spinal cord of newborn mice.

Fig. S5. L-type calcium currents are markedly reduced in neurons of Cav1.3 conditional KO mice.

Data S1. Test statistics and power for data presented in figures.

Data S2. Raw data.

View/request a protocol for this paper from Bio-protocol.

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