Methadone is a local anaesthetic-like inhibitor of neuronal Na\(^+\) channels and blocks excitability of mouse peripheral nerves

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Editor’s key points
- While opioids act mainly via \(\mu\)-receptors, sodium channel activity may contribute to methadone analgesia.
- Methadone and bupivacaine were studied using patch clamp recording in mouse saphenous nerve preparations.
- Methadone unselectively blocks several subtypes of sodium channels with a potency similar to bupivacaine.
- Further work is needed to establish the clinical relevance of this finding.

Background. Opioids enhance and prolong analgesia when applied as adjuvants to local anaesthetics (LAs). A possible molecular mechanism for this property is a direct inhibition of voltage-gated Na\(^+\) channels, which was reported for some opioids. Methadone is an effective adjuvant to LA and was recently reported to inhibit cardiac Na\(^+\) channels. Here, we explore and compare LA properties of methadone and bupivacaine on neuronal Na\(^+\) channels, excitability of peripheral nerves, and cell viability.

Methods. Effects of methadone were explored on compound action potentials (CAP) of isolated mouse saphenous nerves. Patch clamp recordings were performed on Na\(^+\) channels in ND7/23 cells, the \(\alpha\)-subunits Nav1.2, Nav1.3, Nav1.7, and Nav1.8, and the hyperpolarization-activated cyclic nucleotide-gated channel 2 (HCN2). Cytotoxicity was determined using flow cytometry.

Results. Methadone (IC\(_{50}\) 86–119 \(\mu\)M) is a state-dependent and unselective blocker on Nav1.2, Nav1.3, Nav1.7, and Nav1.8 with a potency comparable with that of bupivacaine (IC\(_{50}\) 177 \(\mu\)M). Both bupivacaine and methadone also inhibit C- and A-fibre CAPs in saphenous nerves in a concentration-dependent manner. Tonic block of Nav1.7 revealed a discrete stereo-selectivity with a higher potency for levomethadone than for dextromethadone. Methadone is also a weak blocker of HCN2 channels. Both methadone and bupivacaine induce a pronounced cytotoxicity at concentrations required for LA effects.

Conclusions. Methadone induces typical LA effects by inhibiting Na\(^+\) channels with a potency similar to that of bupivacaine. This hitherto unknown property of methadone might contribute to its high efficacy when applied as an adjuvant to LA.

Keywords: bupivacaine; local anaesthetic; methadone; nerve block; sodium channel

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Analgesic and anaesthetic effects of local anaesthetics (LAs) are primarily due to an inhibition of voltage-dependent Na\(^+\) channels.\(^5,6\) Even though potent and long-lasting LAs are available for clinical use, it is common practice to add adjuvant substances in order to prolong and potentiate the desired LA effect. Recent reports question both efficacy and safety of most substances applied as adjuvants, but state that opioids act as both safe and effective adjuvants.\(^2\) While opioid receptors must be assumed to be essentially involved in mediating this adjuvant effect by opioids, several other opioids have also been demonstrated to exert LA-like effects and to directly inhibit Na\(^+\) channels.\(^5,6\) Strong opioids like fentanyl, sufentanil, and buprenorphine are potent blockers of Na\(^+\) channels, but the concentrations of their clinically used formulations are probably too low for induction of a substantial inhibition of Na\(^+\) channels.\(^5,6\) Morphine, on the other hand, is applied at high concentrations but is a poor Na\(^+\) channel blocker.\(^7\) Here, we explore LA properties of the synthetic opioid methadone, which has been described to be an effective adjuvant when applied in combination with LA in humans.\(^7\) A recent study comparing systemically and epidurally applied methadone in rats suggested an opioid receptor independent site of action for methadone in the spinal cord.\(^8\) Methadone was also demonstrated to induce local anaesthesia in mouse skin.\(^9\) Moreover, we and others recently reported that methadone inhibits the cardiac Na\(^+\) channel Nav1.5 by interacting with the suggested LA-binding site.\(^10,11\) This effect might render methadone its proarrhythmic potential, which is considered
to be associated with cases of sudden cardiac death of patients receiving methadone.12 Another interesting pharmacological property of methadone is a pronounced stereo-selectivity for several target molecules. The inhibition of cardiac hERG K\(^+\) channels is stronger for dextromethadone when compared with levomethadone, an effect which seems to be responsible for the higher proarrhythmic potential of dextromethadone.12 Moreover, dextromethadone seems to be the more potent inhibitor of N-methyl-D-aspartate (NMDA) receptors.13 In contrast, levomethadone is the active stereoisomer for activation of the \(\mu\)-opioid receptor, and it was also reported to inhibit the 5-hydroxytryptamine type 3 receptor more potently when compared with dextromethadone.14 15

As the LA binding is conserved in all \(\alpha\)-subunits of Na\(^+\) channels,16 we hypothesized that methadone might also inhibit neuronal Na\(^+\) channels and thus induce LA. By applying electrophysiological in vitro assays and flow cytometry, we here explore the effects of racemic methadone on neuronal Na\(^+\) channel subunits, on mouse peripheral nerves, and on cell viability. We also explore if levomethadone and dextromethadone exert differential effects on Na\(^+\) channels. Our data essentially demonstrate that methadone is a rather potent but stereoselective blocker of neuronal Na\(^+\) channels, sufficient to inhibit the generation of action potentials in peripheral nerves.

**Methods**

**Cell culture**

All cells were grown under standard tissue culture conditions, that is, 5% CO\(_2\) and 37\(^\circ\)C. Human embryonic kidney (HEK) 293 cells stably expressing Nav1.2 (supplied by Dr Lehmann-Horn, Ulm, Germany), Nav1.3 (supplied by Dr S. Waxman, Department of Neurology, Yale University, New Haven, CT, USA), and Nav1.7 (supplied by Dr A. Lampert, Department of Physiology and Pathophysiology, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany) were cultured in Dulbecco’s modified Eagle medium (DMEM, Gibco-Invitrogen, Germany), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Biochrom, Germany), 1% Taurin, and 1% Geneticin G418 (Gibco-Invitrogen). Human HCN2 (5 \(\mu\)g, a generous gift from Dr J. Stieber, Department of Pharmacology, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany) was transiently expressed in HEK 293 cells by the calcium phosphate precipitation method. Nav1.8 expresses poorly in HEK 293 cells and was therefore investigated in ND7/23 as described previously.17 Neuroblastoma ND7/23 cells were cultured in DMEM supplemented with 10% FBS (Biochrom, Germany), HEPES (2.5%), penicillin/streptomycin (1%, Gibco-Invitrogen), and Taurin (1%, 0.3 M). Green fluorescent protein was co-expressed with HCN2 and Nav1.8 in order to visualize transfected cells. Transfected cells were used for experiments within 2 days.

**Patch clamp technique and data acquisition**

Whole-cell patch clamp recordings were conducted at room temperature. Pipettes (GB150EFT-10, Science Products, Germany) were pulled on an DMZ-Universal Puller (Zeitz, Germany) and heat polished to give a resistance of 1.8–2.2 M\(\Omega\) when filled with the pipette solution. All experiments were conducted using an EPC10 amplifier (HEKA Instruments Inc., NY, USA). Currents were filtered at 5 kHz and sampled at 20–50 kHz. Data were acquired on a PC with the Patchmaster v20 x 60 software (HEKA Instruments Inc.). The series resistance was compensated by 60–80% to minimize voltage errors and the capacitance artifacts were cancelled using the amplifier circuitry. Linear leak subtraction, based on resistance estimates from four hyperpolarizing pulses applied after the depolarization test potential, was used for all protocols except for use-dependent block at 10 Hz. The offset potential was zeroed before the cells were patched. Test solutions were focally applied using a self-made, gravity-driven application system. Only one cell per dish was used. Patch clamp data were analysed with the Pulsefit software (HEKA Instruments Inc.). Curve fitting was performed with Origin 6.0 (Microcal Software, Northampton, MA, USA). Conductance (\(g\)) was determined as \(g = \text{peak current}/(\text{reversal potential} - \text{test pulse voltage})\). To obtain the inactivation curves, peak currents evoked by a test pulse were measured, normalized, and plotted against the conditioning pre-pulse potential. The data were fitted by the Boltzmann equation \(y = y_{\text{max}} \times [1/1 + \exp(\text{EPo} - y)/k]\), where EPo is the voltage of the test pulse, 0.5 the voltage at which \(y = 0.5\), and \(k\) the slope factor. To obtain IC\(_{50}\) values, peak current amplitudes at different drug concentrations were normalized to the value obtained in control solution. The data were fitted with the Hill equation \(y = y_{\text{max}} \times \{\text{IC}_{50^n}\times y\}^{n} / (\text{IC}_{50^n} + y\}\), where \(y_{\text{max}}\) is the maximal amplitude, IC\(_{50}\) the concentration at which \(y_{\text{max}} / y = 0.5\), and \(n\) the Hill coefficient.

The bath solution for recordings of Na\(^+\) channels contained (in mM): 140 NaCl, 3 KCl, 1 MgCl\(_2\), 1 CaCl\(_2\), 10 HEPES, and 10 glucose. For recordings of Nav1.8 in ND7/23 cells, 300 nM tetrodotoxin (TTX, Alomone Labs, Israel) was included in the bath solution in order to block all endogenous TTX-sensitive Na\(^+\) channels. The pipette solution contained (in mM): 140 CsF, 10 NaCl, 1 EGTA, and 10 HEPES. The bath solution for recordings of HCN2 contained (in mM): 120 mM NaCl, 20 mM KCl, 1 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), 10 mM HEPES, and 10 mM glucose. The pipette solution contained (in mM): 10 mM NaCl, 30 mM KCl, 90 mM potassium aspartate, 1 mM MgSO\(_4\), 5 mM EGTA, and 10 mM HEPES. Pipette and bath solutions were adjusted to pH 7.4. The osmolarity of all solutions was adjusted to 290–300 mosm. All solutions were stored at 4\(^\circ\)C. Errors resulting from liquid junction potentials were not corrected for.

**Chemicals**

Racemic methadone, dextromethadone, and bupivacaine were purchased from Sigma-Aldrich (Germany) and prepared as stock solutions in DMSO. Levomethadone was obtained from Sanofi-Aventis (L-Polamidon®, Sanofi-Aventis, Germany). Stock solutions were stored at 4\(^\circ\)C and test solutions for experiments were prepared directly before patch clamp recordings.

**Compound action potential recordings**

An isolated saphenous nerve in vitro preparation from the mouse was used to record compound action potentials (CAPs)
Methadone is an LA-like inhibitor of neuronal Na\(^+\) channels by methadone. (A) Representative families of current traces generated by Na\(^+\) channels in ND7/23 cells in the presence of control solution or 100 \(\mu\)M methadone. Cells were held at -120 mV and currents were elicited by 20 ms long test-pulses ranging from -90 to 40 mV in steps of 10 mV. Concentration-dependent tonic block of resting Na\(^+\) channels by methadone. Cells were held at -120 mV, inactivated channels were induced by a 10 s long pre-pulse at -70 mV followed by a 100 ms long pulse at -120 mV allowing recovery from fast inactivation. Peak amplitudes of Na\(^+\) currents at different drug concentrations were normalized with respect to the peak amplitude in control solution and plotted against the concentration of methadone. Data were fitted with the Hill equation represented by the solid line. (C) Development of use-dependent block of neuronal Na\(^+\) channels by 10 and 100 \(\mu\)M methadone. Peak currents were normalized to the amplitude of the first pulse and plotted against the pulse number. (D) Normalized current–voltage curves of currents obtained in (A). Currents were normalized to the peak currents amplitude and plotted against the corresponding membrane potential. The lines are drawn to guide the eye. (E) Voltage-dependency of fast inactivation of Na\(^+\) channels in ND7/23 cells in control solution and in the presence of 3 \(\mu\)M methadone. Fast inactivation was induced by 50 ms long test-pulses ranging from -120 to -30 mV, and the remaining fraction of available channels was examined with a 20 ms long pre-pulse to 0 mV. The solid lines represent fits calculated with the Boltzmann equation. (F) Voltage-dependency of slow inactivation of Na\(^+\) channels by 10 and 100 \(\mu\)M methadone. Slow inactivation was induced by 10 s long pre-pulses ranging from -120 to -10 mV in steps of 10 mV followed by a 100 ms long pre-pulse at -120 mV allowing recovery and calculated as described above for methadone. (G) Concentration-dependent tonic block of resting Na\(^+\) channels by 50 \(\mu\)M bupivacaine with and without 10 \(\mu\)M methadone. Peak currents of neuronal Na\(^+\) channels in ND7/23 cells in control solution and in the presence of 3 \(\mu\)M bupivacaine with and without 10 \(\mu\)M methadone. (H) Fits calculated with the Boltzmann equation. (I) Use-dependent block of neuronal Na\(^+\) channels in ND7/23 cells in control solution and in the presence of 10 \(\mu\)M methadone. Peak currents were normalized to the amplitude of the first pulse and plotted against the pulse number. All data are presented as mean (SD).
Results

Methadone is a state-dependent blocker of neuronal Na\(^+\) channels

We first asked if racemetic methadone is able to block neuronal Na\(^+\) channels. The neuroblastoma cell line ND7/23 expresses several \(\alpha\)-subunits of sensory neuronal Na\(^+\) channels and can thus serve as a cellular model for sensory neurones which express several different \(\alpha\)-subunits of Na\(^+\) channels.\(^{19}\) As is illustrated in Figure 1A, 100 \(\mu\)M methadone induces a strong reduction in the peak current amplitude of Na\(^+\) currents activated by a series of pulses ranging from \(-90\) to \(60\) mV in cells held at \(-120\) mV [59 (3\%) block, \(n=9\)]. Next, the concentration-dependency of tonic block was examined for resting and inactivated Na\(^+\) channels in ND7/23 cells. Resting channels were explored in cells held at \(-120\) mV by test pulses to 0 mV applied at 0.1 Hz. Methadone induces a concentration-dependent block with an IC\(_{50}\) value of 134 (4) \(\mu\)M [Hill coefficient \(-1.5 (0.1), n=8, \text{Fig. 1a}\)]. The block of inactivated channels was examined by a protocol consisting of a 10 s long pre-pulse to \(-70\) mV followed by 100 ms long pulse at \(-120\) mV allowing recovery from fast inactivation, and finally, a test pulse to 0 mV. As is demonstrated in Figure 1a, the induction of inactivated channels results in an enhanced tonic block by methadone [IC\(_{50}\) 70 (5) \(\mu\)M, Hill coefficient \(-1.2 (0.1), n=11\) \((P<0.001, \text{unpaired t-test when compared with resting channels})\). A typical inhibitory property of classical LA on Na\(^+\) channels is a use-dependent block. When Na\(^+\) currents in ND7/23 cells were stimulated at 10 Hz in cells held at \(-120\) mV, we observed a concentration-dependent use-dependent block by 10 \(\mu\)M [7 (2\%) block at the last pulse, \(n=8\)] and 100 \(\mu\)M [39 (10\%) block at the last pulse, \(n=8\), Fig. 1c]. Na\(^+\) currents did not display a prominent shift in the current–voltage relationship when 100 \(\mu\)M methadone was applied (Fig. 1d). Accordingly, 100 \(\mu\)M methadone did not induce a significant shift of the calculated midpoints (\(V\)\(_{1/2}\)) of the conductance [control: \(V\)\(_{1/2}\) \(-27 (1)\) mV; 100 \(\mu\)M methadone: \(V\)\(_{1/2}\) \(-30 (1)\) mV, \(n=12\) \((P>0.5, \text{paired t-test})\). However, we find that methadone stabilized both fast inactivation and slow inactivation. Fast inactivation was induced by 50 ms long pre-pulses ranging from \(-120\) to \(-30\) mV in steps of 5 mV, and the remaining fraction of available channels was examined with a 20 ms long pre-pulse to 0 mV. As is demonstrated in Figure 1e, methadone induces a concentration-dependent shift of the voltage-dependent fast inactivation [control: \(V\)\(_{1/2}\) \(-67 (0.1)\) mV; 10 \(\mu\)M: \(V\)\(_{1/2}\) \(-73 (0.1)\) mV; 100 \(\mu\)M: \(V\)\(_{1/2}\) \(-78 (0.2)\) mV, \(n=12\) \((P<0.01 \text{ for both concentrations, repeated-measures analysis of variance (ANOVA), Tukey's post hoc test})\). Slow inactivation was induced by 10 s long pre-pulses ranging from \(-120\) to \(-10\) mV in steps of 10 mV followed by a 100 ms long inter-pulse at \(-120\) mV allowing recovery from fast inactivation. A prominent shift of the voltage-dependency of slow inactivation was induced already by 3 \(\mu\)M methadone [control: \(V\)\(_{1/2}\) \(-51 (1)\) mV; 3 \(\mu\)M: \(-60 (1)\) mV, \(n=11, P<0.01, \text{paired t-test, Fig. 1r}\). Taken together, these data suggest that methadone is a rather potent and state-dependent inhibitor of neuronal Na\(^+\) channels. We next compared this property of methadone with that of bupivacaine, which is a potent LA frequently applied for regional anaesthesia in clinical practice. Tonic block of resting Na\(^+\) channels in ND7/23 cells by bupivacaine was examined as described above for methadone. Bupivacaine induces a concentration-dependent block with an IC\(_{50}\) value of 133 (7) \(\mu\)M [Hill coefficient \(-1.1 (0.1), n=7, \text{Fig. 1a}\)], that is, with a similar potency as was determined for methadone. The same protocol was used to examine if the co-application of bupivacaine and methadone results in an abbreviated, additive, or potentiated inhibition. As is depicted in Figure 1h, the addition of methadone to 50 \(\mu\)M bupivacaine [26 (4\%) N, \(n=9\)] results in an increased block reflecting an additive effect; 10 \(\mu\)M methadone: 30 (5\%) and 100 \(\mu\)M methadone: 56 (5\%), \(n=9, P<0.001\) for both concentrations, repeated-measures ANOVA, Tukey's post hoc test. We also examined if the addition of a low concentration of methadone to bupivacaine results in an increased use-dependent block. While 10 \(\mu\)M bupivacaine induces a stronger use-dependent block at 10 Hz [20 (4\%), \(n=11\)] when compared with 10 \(\mu\)M methadone alone \((P<0.001, \text{unpaired t-test})\), the combination of 10 \(\mu\)M of both substances does not result in a significantly enhanced use-dependent block [21 (5\%), \(n=11, P>0.05, \text{paired t-test, Fig. 1l}\).

Methadone is an unselective blocker of neuronal Na\(^+\) channels

The excitability of peripheral sensory axons and dorsal horn neurones is dictated by an array of \(\alpha\)-subunits of Na\(^+\) channels. While the excitability of C-fibres is largely carried by the subunits Nav1.7 and Nav1.8,\(^{20} 21\) dorsal horn neurones predominantly express Nav1.2 and Nav1.3.\(^{22}\) We therefore studied tonic and use-dependent block by methadone on these \(\alpha\)-subunits in order to identify possible subunit-specific effects of methadone. Again, tonic block by increasing concentrations of racemetic methadone was examined on currents activated at 0.1 Hz by 10 ms (Nav1.2, Nav1.3, Nav1.7) or 50 ms (Nav1.8) long test pulses to 0 mV in cells held at \(-120\) mV (Fig. 2a, Nav1.8). As is demonstrated in Figure 2b, we did not observe any substantial subunit-selective differences with regard to tonic block of resting channels [IC\(_{50}\) Nav1.2: 86 (5) \(\mu\)M \((n=10)\); Nav1.3 119 (5) \(\mu\)M \((n=10)\); Nav1.7 102 (5) \(\mu\)M \((n=10)\); Nav1.8 112 (2) \(\mu\)M \((n=10)\) \((P>0.05, \text{ANOVA, Tukey's post hoc test})\). As demonstrated by a typical recording on Nav1.7 in Figure 2c, we also investigated use-dependent block induced by 100 \(\mu\)M methadone at 10 Hz on all four subunits. As is shown in Figure 2d, methadone induces a similar use-dependent block on Nav1.2 [29 (11\%), \(n=7\), Nav1.3 [23 (5\%), \(n=11\), Nav1.7 [31 (4\%), \(n=10\)], and Nav1.8 [35 (10\%), \(n=8\)]. While Nav1.3 was found to exert a significantly smaller use-dependent inhibition when compared with both Nav1.7 and Nav1.8 \((P<0.05, \text{ANOVA, Tukey's post hoc test})\), the other subunits did not display different use-dependent block by methadone \((P>0.05, \text{ANOVA, Tukey's post hoc test})\).
Methadone reversibly blocks nerve conduction of C- and A-fibres in peripheral nerve axons

In order to explore if racemic methadone is indeed able to directly inhibit excitability of peripheral nerves, we next used isolated mouse saphenous nerves for CAP recordings. Electrically evoked CAPs of C- and A-fibres from the same nerve were recorded as described previously. Application of increasing concentrations of methadone (30–1000 \(\mu M\)) instantaneously resulted in a concentration-dependent and reversible reduction of both C- (Fig. 3A lower) and A-fibre (Fig. 3B lower) amplitudes. The concentration-dependency of this reduction in amplitudes is illustrated by dose–response curves (Fig. 3D), and the IC_{50} values for this inhibitory effect were 273 (3) \(\mu M\) for C-fibres and 594 (5) \(\mu M\) for A-fibres (both \(n=7\)). Moreover, we observed a concentration-dependent increase in latency for both C- (Fig. 3A upper) and A-fibre CAPs (Fig. 3B upper), which was reversible during the subsequent wash-out phase. As is shown by dose–response curves in Figure 3C, the IC_{50} values for this effect were 570 (6) \(\mu M\) for C-fibres and 634 (7) \(\mu M\) for A-fibres (both \(n=7\)). Again, we performed identically designed experiments using racemic bupivacaine (10–1000 \(\mu M\)) in order to compare the efficacy of methadone with an established and potent LA. As becomes obvious in dose–response curves for the decrease in C- and A-fibre CAP amplitudes [IC_{50} C-fibre: 133 (23) \(\mu M\); A-fibre: 168 (6) \(\mu M\), both \(n=7\); Fig. 3D] and the increase in CAP latencies of C- and A-fibres [IC_{50} C-fibre: 189 (4) \(\mu M\); A-fibre: 239 (2) \(\mu M\), both \(n=7\); Fig. 3C], bupivacaine showed to be more potent than racemic methadone in blocking excitability and conduction of peripheral nerves.

Stereo-selective effects of methadone on Nav1.7

We next investigated if dextromethadone and levomethadone, for which the molecular structures are depicted in Figure 4A, interact with Nav1.7 in a stereo-selective manner. As is demonstrated in Figure 4B, levomethadone [IC_{50} 63 (10) \(\mu M\), \(n=10\)] induced a significantly stronger tonic block of resting Nav1.7 channels expressed in HEK 293 cells when compared with dextromethadone [IC_{50} 86 (13) \(\mu M\), \(n=10\)] (\(P<0.05\), unpaired t-test). Although this stereo-selective difference reached statistical significance, it seems rather unlikely that a \(\sim 1.4\)-fold stronger potency for levomethadone will be of great relevance. Accordingly, this...
stereo-selective tonic inhibition did not reach significance on inactivated Nav1.7 channels \( [IC_{50} \text{ levomethadone 42 (3) } \mu M, n=10; \text{ dextromethadone 53 (6) } \mu M, n=10] \) \( (P>0.5, \text{ unpaired } t\text{-test}) \). Moreover, use-dependent block of Nav1.7 at 10 Hz was not different for levomethadone \( (38 (11)\% , n=7) \) and dextromethadone \( (36 (7)\% , n=10) \) \( (P>0.5, \text{ unpaired } t\text{-test}) \).

**Methadone is not a potent inhibitor of HCN2**

As already mentioned above, several substances are used as adjuvants for LAs. Recent reports argue that the adjuvant effects induced by the \( \alpha_2 \)-receptor agonists clonidine and dexmedetomidine are primarily due to an inhibition of hyperpolarization-activated cyclic nucleotide-gated channels (HCN). \( 23 \) \( 24 \) HCN channels are also inhibited by LA and opioids. \( 25 \) \( 26 \) thus, they are possibly important sites of action for both LA and several adjuvants used in clinical practice. We therefore explored the effects of racem tic methadone on HCN2, one of the most prevalent HCN channel isoforms in C-fibres. \( 27 \) HCN2 inward currents were activated by 1 s long test pulses to 100 mV applied at 0.1 Hz in cells held at \(-40\) mV (Fig. 5a). The inhibitory effect by racem tic methadone was quite poor when compared with the effects on neuronal Na\(^+\) channels \( [IC_{50} \text{ 891 (233) } \mu M, n=7, \text{ Fig. 5a}] \).

**Methadone and bupivacaine induces concentration-dependent apoptosis and necrosis**

More or less, all LAs induce a concentration-dependent cytotoxicity, a property which is regarded to be responsible for transient and persistent neurological symptoms occurring after application of LA in clinical practice. \( 28 \) We therefore asked if methadone induces cytotoxicity at clinically relevant concentrations. For this purpose, untransfected HEK 293t cells were examined by flow cytometry after treatment with increasing concentrations of methadone or bupivacaine for 12 h. Methadone induces a concentration-dependent cytotoxicity in HEK 293t cells at concentrations below 1 mM (Fig. 6a). As is depicted in Figure 6, the percentage of cells being positive for Annexin V (i.e. early apoptosis) was 4 (3\%) \( (n=5) \) for untreated cells and increased to 11 (10\%) \( (n=5) \) after treatment with 750 \( \mu M \) methadone. Moreover, cells being positive for both Annexin V and PI (i.e. necrosis) increased from 8 (5\%) \( (n=5) \) for untreated cells to 44 (21\%) for cells treated with 750 \( \mu M \) methadone.
LA are the most frequently studied and best characterized Na\(^+\) channel inhibitors. LA inhibit Na\(^+\) channels by interacting with conserved intracellular residues of all \(\alpha\)-subunits, and this interaction obeys a strong state-dependency.\(^6\) While resting Na\(^+\) channels are poorly blocked, LA have a high affinity to inactivated and open channels.\(^6\) In this study, we demonstrate that methadone induces typical LA-like effects on neuronal Na\(^+\) channels, that is, it inhibits Na\(^+\) channels in a strongly state-dependent manner. This property correlates very well with the fact that methadone was shown to inhibit Nav1.5 by interacting with the LA-binding site.\(^1\) The fact that methadone does not really discriminate between Nav1.2, Nav1.3, Nav1.7, and Nav1.8 probably reflects the fact that the LA-binding site is conserved in all \(\alpha\)-subunits.\(^6\) This interaction of methadone with neuronal Na\(^+\) channels is not novel among opioids, that is, several other opioids have been described to be state-dependent inhibitors of neuronal Na\(^+\) channels and to interact with the LA-binding site.\(^5\) In contrast to most other strong opioids, however, clinically used formulations of methadone are applied at concentrations (10 mg ml\(^{-1}\)=32.3 mM)\(^10\) likely to be high enough to induce a relevant inhibition of Na\(^+\) channels even after tissue distribution. We find that 1 mM methadone completely blocks CAP in C- and A-fibres in mouse peripheral nerves, and that lower concentrations reduced both conduction velocity and the amplitudes of CAP. Even though methadone was the more potent tonic Na\(^+\) channel blocker when compared with bupivacaine, we find that bupivacaine was considerable more potent in abrogating excitability of mouse peripheral nerves. This discrepancy between the effects on single cells and intact nerves may be due to the higher lipophilicity of bupivacaine when compared with methadone (octanol:water partition coefficient 365 and 117, respectively), allowing a better permeation of bupivacaine through the perineural barrier of intact nerves. Nevertheless, methadone
applied in the epidural space alone or together with LA was reported to induce sufficient analgesia in patients during and after surgery.\textsuperscript{8,11} Even though the activation of the \(\mu\)-opioid receptor may almost entirely account for this analgesic effect of methadone, there is rather convincing evidence indicating that a direct LA action of methadone may indeed be of relevance: (i) Haroutiunian and colleagues\textsuperscript{8} recently reported that epidural methadone induces stronger antinociceptive effects and less supraspinal side-effects when compared with systemically administered methadone in rats. As both routes of administration resulted in similar plasma levels of methadone, the authors concluded that methadone is likely to interact with an opioid receptor-independent mechanism in the spinal cord.\textsuperscript{8} (ii) Although dextromethadone almost completely fails to activate opioid receptors, it was demonstrated to be antinociceptive when applied epidurally in rats with the formalin test.\textsuperscript{32} (iii) Methadone induces analgesia when applied topically on mouse skin.\textsuperscript{9} Although none of these points really prove that the inhibition of Na\(^{+}\) channels is of relevance for regional anaesthesia and LA in clinical practice, our finding that methadone acts as a quite potent LA on neuronal Na\(^{+}\) channels identifies a very plausible opioid receptor-independent mechanism for analgesia and anaesthesia induced by methadone. We did not observe any substantial stereo-selectivity for tonic or use-dependent block of Nav1.7 by dextromethadone and levomethadone, that is, the interaction of methadone with Na\(^{+}\) channels seems to differ from the interaction of methadone with several other membrane proteins. While levomethadone is a potent \(\mu\)-opioid receptor agonist, dextromethadone almost completely lacks opioidergic properties and hardly induces any analgesia when administered systemically.\textsuperscript{14} Although not with such a pronounced stereo-selectivity, dextromethadone is the more potent inhibitor of cardiac hERG K\(^{+}\) channels (IC\(_{50}\) \(\approx\) 12 \(\mu\)M).\textsuperscript{12} This property is regarded as the main mechanism for the proarrhythmic potential of racemate methadone supposed to be responsible for the initiation of long QT syndromes and cases of sudden cardiac death in patients receiving high dosages of methadone.\textsuperscript{12} Dextromethadone was also reported to be the more potent inhibitor of NMDA receptors; however, this stereo-selectivity was marginal and both isomers inhibited NMDA receptors at low micromolar concentrations (IC\(_{50}\) \(\approx\) 4–20 \(\mu\)M).\textsuperscript{13}

To our knowledge, the ideal pharmacological profile of an effective and safe adjuvant for LA has not yet been defined. However, the pharmacological properties of levomethadone might indeed make it an interesting and thus clinically useful adjuvant. The activation of \(\mu\)-opioid receptors and simultaneous inhibition of NMDA receptors and Na\(^{+}\) channels should make levomethadone an effective adjuvant for neuraxial blocks applied alone or in combination with LA. In addition, we also find that methadone is a weak blocker of HCN2. While at this stage we can only speculate about the relevance of this effect, HCN channels were suggested to be important targets for the adjuvant effects of both clonidine and dexametadomidine.\textsuperscript{23,24}

It is also tempting to speculate about the relevance of an inhibition of Na\(^{+}\) channels when methadone is applied systemically for treatment of chronic and neuropathic pain. We recently demonstrated that buprenorphine is a potent Na\(^{+}\) channel blocker and suggested that this property might be responsible for its pronounced antihyperalgesic property.\textsuperscript{5,33} This antihyperalgesic property of buprenorphine stands in contrast to many other clinically used opioids known to induce hyperalgesia instead of antihyperalgesia.\textsuperscript{33} Even though the literature is conflicting as to whether methadone prevents opioid-induced hyperalgesia or not,\textsuperscript{36} it seems reasonable that the rather potent inhibition of Na\(^{+}\) channels by methadone might counteract hyperalgesia.

Our study also demonstrates that methadone induces cytotoxicity at high micromolar concentrations, a property which has also been demonstrated for more or less all clinically used LA.\textsuperscript{28,35} This LA-induced neurotoxicity can induce nerve
Methadone is an LA-like inhibitor

_damage resulting in transient or even chronic neurological symptoms such as the cauda equine syndrome._

Recent reports have indeed demonstrated that some commonly applied adjuvants to LA also induce neurotoxicity and thus increase LA-induced neurotoxicity.36 37 Such reports raise the question as to whether adjuvants might be responsible for an increased incidence of LA-induced neurological side-effects.

In the case of methadone, it was reported to induce both demyelination and necrosis in the spinal cord when administered intrathecally in rats.38 39 Thus, the cytotoxic potential of methadone should give rise to concern when it is applied as an adjuvant to LA or used for epidural or intrathecal analgesia.

Our data demonstrate for the first time that methadone acts as a potent LA on neuronal Na$^{+}$ channels, as a blocker of nerve conduction of C- and A-fibres in peripheral nerves, and reduces the viability of cultured cells. Together with the activation of μ-opioid receptors and inhibition of NMDA receptors, this pharmacological property of methadone might explain why it is effective when used as an adjuvant to LA and when administered alone intrathecally or epidurally to induce analgesia and anaesthesia. The clinical use of methadone as an adjuvant, however, might carry the risk of neurological side-effects due to neurotoxicity.

Authors’ contributions
C.S.: patch clamp data collection, wrote the manuscript. K.K.: compound action potential data collection. T.S.: flow cytometry data collection. M.W.: patch clamp data collection. V.S.: patch clamp data collection. N.F.: data analysis, data collection. F.W.: data analysis, editing of the manuscript. J.A.: data analysis, editing of the manuscript. A.L.: design of the study, writing and editing of the manuscript.

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Declaration of interest
None declared.

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References
1 Fozzard HA, Sheets MF, Hanck DA. The sodium channel as a target for local anaesthetic drugs. _Front Pharmacol_ 2011; 2: 68
14 Kristensen K, Christensen CB, Christrup LL. The mu1, mu2, delta, kappa opioid receptor binding profiles of methadone stereoisomers and morphine. Life Sci 1995; 56: PL45 – 50
16 Leffler A, Reckzeh J, Nau C. Block of sensory neuronal Na+ channels by the secreolytic ambroxol is associated with an interaction with local anaesthetic binding sites. Eur J Pharmacol 2010; 630: 19 – 28
17 Leffler A, Reiprich A, Mohapatra DP, Nau C. Use-dependent block by lidocaine but not amitriptyline is more pronounced in tetrodotoxin (TTX)-Resistant Nav1.8 than in TTX-sensitive Na+ channels. J Pharmacol Exp Ther 2007; 320: 354 – 64
19 Leffler A, Reckzeh J, Nau C. Block of sensory neuronal Na+ channels by the secreolytic ambroxol is associated with an interaction with local anaesthetic binding sites. Eur J Pharmacol 2010; 630: 19 – 28
25 Emery EC, Young GT, Berrocoso EM, Chen L, McNaughton PA. HCN2 ion channels play a central role in inflammatory and neuropathic pain. Science 2011; 333: 1462 – 6
27 Wagner LE II, Eaton M, Sabnis SS, Gingrich KJ. Meperidine and lido- caine block of recombinant voltage-dependent Na+ channels: evidence that meperidine is a local anaesthetic. Anesthesiology 1999; 91: 1481 – 90
30 Scott CC, Robbins EB, Chen KK. Pharmacologic comparison of the optical isomers of methadon. J Pharmacol Exp Ther 1948; 93: 282 – 6
32 Williams BA, Hough KA, Tsui BY, Ibinson JW, Gold MS, Gebhart GF. Neurotoxicity of adjuvants used in perineural anaesthesia and an- tihyperalgesia. Br J Anaesth 2004; 93: 1481 – 90
37 Williams BA, Hough KA, Tsui BY, Ibinson JW, Gold MS, Gebhart GF. Neurotoxicity of adjuvants used in perineural anaesthesia and an- tihyperalgesia in comparison with ropivacaine. Reg Anesth Pain Med 2011; 36: 225 – 30

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