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# Spinal calmodulin inhibitors reduce *N*-methyl-D-aspartate- and septide-induced nociceptive behavior

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**Abstract**

The effect of two calmodulin inhibitors, W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphtalenesulfonamide) and calmidazolium, on the nociceptive behavior induced by the intrathecal injection of NMDA (*N*-methyl-D-aspartate), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) or of septide is described. Lumbar intrathecal injection of NMDA, AMPA or septide induced a caudally directed nociceptive reaction (biting, scratching and licking). The nociceptive behavior induced by NMDA (4 μg) was dose dependently inhibited when W-7 (0.25–1 μmol/rat) or calmidazolium (0.12–0.5 μmol/rat) was coinjected. Biting, scratching and licking produced by AMPA (2 μg) were unaffected by intrathecal calmodulin inhibitors. Finally, septide-evoked nociceptive behavior (2 μg) was antagonized by W-7 (0.12–0.5 μmol/rat) and calmidazolium (0.06–0.25 μmol/rat). Thus, calmodulin inhibitors prevent the nociceptive reaction evoked by drugs that modify intracellular $Ca^{2+}$, NMDA and septide, without affecting the nociceptive response induced by AMPA, for which $Ca^{2+}$ is not the main second messenger. © 1997 Elsevier Science B.V.

*Keywords:* Pain; Analgesia; Calmodulin inhibitor; Spinal cord; NMDA (*N*-methyl-D-aspartate); AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid); Septide

## 1. Introduction

Several types of excitatory and inhibitory mediators are involved in the transmission of nociceptive messages in the spinal cord. Among the molecules most directly involved as excitatory neurotransmitters are two major families: excitatory amino acids, such as glutamate and aspartate, acting through AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and/or NMDA (*N*-methyl-D-aspartate) receptors, and neurokinins, mainly substance P and neurokinin A, acting through tachykinin $NK_1$ and tachykinin $NK_2$ receptors, respectively (Fleetwood-Walker et al., 1990). Accordingly, the release in the spinal cord of excitatory amino acids (Skilling et al., 1988) and neurokinins (Go and Yaksh, 1987) in response to a noxious stimulation has been demonstrated. Furthermore, spinal administration of substance P (Henry, 1976; Murase and Randic, 1984), neurokinin A (Fleetwood-Walker et al., 1993) and excitatory amino acids (Willcokson et al., 1984; Aanonsen et al., 1990) activates sensitive spinal neurons, as seen in electrophysiological assays.

In behavioral studies, the intrathecal (i.t.) injection of low doses of some of these molecules can induce hyperalgesic responses to noxious stimuli in both mice and rats. This has been reported for neurokinin A (Fleetwood-Walker et al., 1990), substance P (Matsumura et al., 1985), selective agonists of tachykinin $NK_1$ and tachykinin $NK_2$ receptors (Picard et al., 1993), glutamate and aspartate (Coderre and Melzack, 1992). When higher doses of these molecules are injected i.t., they themselves induce nociceptive stimulation, producing aversive behavior consisting of biting, scratching and/or licking of the corresponding dermatomes (Wilcox, 1988). This has been repeatedly demonstrated with selective agonists of tachykinin $NK_1$, AMPA and NMDA receptors in both mice (Hylden and Wilcox, 1981; Aanonsen and Wilcox, 1987) and rats (Papir-Kricheli et al., 1987; Björkman et al., 1994). However, nociceptive behavior induced by tachykinin $NK_2$ receptor agonists has been seen in mice (Gamse and Saria, 1986; Ravard et al., 1994), but not in rats. Due to their potential ability to induce analgesia, considerable effort has been focussed on the design of new and selective antagonists for excitatory amino acids and neurokinin receptors. Recent reports describe analgesic responses produced by the administration of selective antagonists of

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NMDA receptors (Coderre and Melzack, 1992) and tachykinin $NK_1$ and $NK_2$ receptors (Sakurada et al., 1993, Seguin et al., 1995).

Recently, we have reported that the i.t. injection of two calmodulin inhibitors, W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphtalenesulfonamide) and calmidazolium, induces analgesia in the formalin and the tail-flick tests in rats (Menéndez et al., 1996; Menéndez and Baamonde, 1996). Calmodulin is an intracellular protein which triggers many $Ca^{2+}$-related effects (Gnegy, 1993). Consequently, some of the intracellular effects of $Ca^{2+}$ can be prevented by the inhibition of calmodulin. Related to spinal neurotransmitters involved in nociception, NMDA receptors are ligand-gated $Ca^{2+}$ channels and tachykinin $NK_1$ and $NK_2$ receptors increase intracellular $IP_3$ (1,4,5-inositol triphosphate) levels which, in turn, release $Ca^{2+}$ from intracellular stores (Ghosh and Greenberg, 1995). Thus, the stimulation of both NMDA and tachykinin receptors triggers cellular signals that probably involve calmodulin activation.

We have tested the effects of two standard calmodulin inhibitors (W-7 and calmidazolium) injected i.t. on the nociceptive reaction produced by spinal administration of AMPA, NMDA and tachykinin $NK_1$ receptor agonists in rats. We also tried testing the effects of calmodulin inhibitors on the response evoked by one tachykinin $NK_2$ receptor agonist, but, as described in Section 3, no nociceptive response was obtained with this drug.

## 2. Material and methods

### 2.1. Animals

Male Wistar rats ($N = 202$), weighing 250–350 g, from the Animalario de la Universidad de Oviedo (Reg. 33044 13A) exposed to a light–dark cycle of 12 h and with water and food 'ad libitum', were used. The experiments were performed between 9 and 14 h in a room with controlled humidity (60%), temperature (20–22°C) and noise. Rats were randomly assigned to each group and received an injection of the corresponding drugs or solvents. Animals were used only once and killed at the end of the experiment by overexposure to ether.

### 2.2. Drugs

W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphtalenesulfonamide; Sigma) was dissolved in distilled water (50 μl) and calmidazolium (R 24571; 1-[bis(*p*-chlorophenyl)methyl]-3-[2,4-dichloro-β-(2,4-dichlorobenzyloxiphenethyl] imidazolinium chloride; Sigma) was dissolved in 50 μl of 10% DMSO (dimethyl sulfoxide, Sigma). NMDA (*N*-methyl-D-aspartate; Sigma), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Tocris-Cookson) and septide ([$pGlu^6$, $Pro^9$] substance P-(6-11); Sigma) were coinjected with W-7 or calmidazolium in their corresponding solvents. [$Ala^5$,β-$Ala^8$]α-neurokinin-(4-10) was injected alone dissolved in 50 μl of water.

Intrathecal injections were given by direct lumbar puncture according to a slightly modified version of a method previously used by us (Menéndez et al., 1996; Menéndez and Baamonde, 1996) and described by Papir-Kricheli et al. (1987). The rats were lightly anesthetized with ether and a 1 to 2 cm cut was then made in the dorsal lumbar skin. The needle of a hypodermic syringe ($0.33 \times 13$ mm) was introduced between the L4 and L5 vertebrae. The whole procedure lasted about 2–3 min. As soon as behavioral symptoms of recovery from anesthesia (usually 1–3 min) were observed the liquid was slowly injected.

### 2.3. Nociceptive assays

Immediately after the i.t. injection of the excitatory agent with or without the corresponding calmodulin inhibitor, the rats were carefully placed in a transparent plastic cage ($45 \times 25 \times 20$ cm), in which they were observed during a period of 15 min. The time spent in biting, scratching or licking the hindquarters was, at least, 15 min in the control rats treated with NMDA, AMPA or septide. This behavior was recorded with a chronometer for three successive periods of 5 min. Only occasionally, was the scratching behavior not strictly confined to the posterior part of the body, including anterior regions. Neither the various doses of calmidazolium or W-7 nor the solvents used, themselves induced nociceptive behavior, consistent with our earlier observations (Menéndez et al., 1996; Menéndez and Baamonde, 1996).

### 2.4. Data analysis

The means of the time spent in nociceptive behavior and their standard errors were calculated for each group. In one experiment, data are referred to the total period of observation (15 min) and for the rest, three successive periods of 5 min were considered. Comparisons were made by using an initial one-way analysis of variance (ANOVA) for control and experimental groups. This was followed by the Newman–Keuls test to calculate the significance of individual differences among groups at each time studied. The level of significance was set at $P < 0.05$. In order to quantify the potency ratio of both calmodulin inhibitors, a quantal $ED_{50}$ value (and its corresponding 95% confidence limits; C.L.) was calculated according to Litchfield and Wilcoxon (1949) with a computer program described by Tallarida and Murray (1987). For this analysis, an animal was considered to show analgesia only when the total time spent biting, scratching or licking by the animal reached, at least, the 75% inhibited value for the corresponding control group. In this way, the $ED_{50}$ represents the dose of calmodulin inhibitors able to induce a clear-cut analgesic effect in the 50% of the animals studied.

## 3. Results

### 3.1. Nociceptive behavior induced by the i.t. injection of NMDA, AMPA, septide and [Ala$^5$,β-Ala$^8$]α-neurokinin-(4-10)

Intrathecal NMDA (0.5–8 μg), AMPA (0.5–4 μg) and septide (0.5–4 μg) induced dose-related biting, scratching and licking (Fig. 1). The nociceptive behavior caused by the three drugs started immediately after their i.t. injection, but the behavior induced by each drug was not exactly the same.

The i.t. administration of NMDA induced a vigorous nociceptive reaction accompanied by closed eyes, piloerection and, more rarely, vocalization, probably reflecting pain. Also, NMDA often induced motor excitation, sometimes resulting in restlessness and spontaneous myoclonic twitches of the tail, that disappeared with time. NMDA was also the only drug that induced true biting behavior, such that the injection of 4 or 8 μg of NMDA produced self-injury of the tail in some cases. This behavior was never observed with the other agonists studied. Since 4 μg induced a near-maximal response, this dose was selected for further experiments.

Intrathecal AMPA seems to induce a more general excitation simultaneously with the nociceptive reaction. This could explain why increasing the dose from 2 to 4 μg reduced rather than increased the time spent biting, scratching and licking. With 4 μg injected, motor restlessness was intense and accompanied by difficulties in breathing. For this reason, the dose of 2 μg was chosen to test the effect of calmodulin inhibitors.

Septide produced a clear-cut and well localized nociceptive behavior. The rats showed no motor excitation. Since 2 μg induced a maximal response, this dose was selected to test the effect of calmodulin inhibitors.

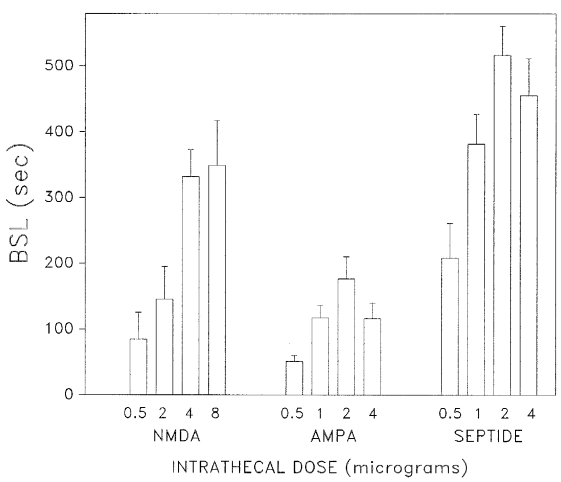


Fig. 1. Time spent biting, scratching, licking (nociceptive behavior) after intrathecal administration of NMDA (0.5–8 μg), AMPA (0.5–4 μg) and septide (0.5–4 μg) in rats. The nociceptive behavior was measured during the 15 min immediately after the injection of the agonists. The means and corresponding standard errors are presented ($n = 5–7$).

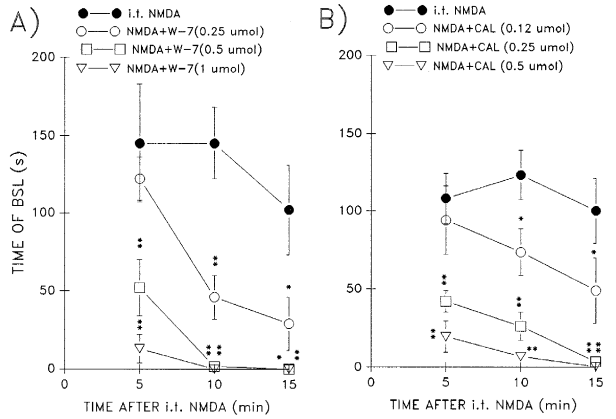


Fig. 2. Effect of i.t. W-7 (0.25–1 μmol/rat) (A) and calmidazolium (CAL, 0.12–0.5 μmol/rat) (B) on the nociceptive behavior induced by 4 μg of i.t. NMDA. The means and corresponding standard errors are given for three 5-min periods after the intrathecal injection. Comparisons were made for each time between the effect of NMDA alone and in the presence of calmodulin inhibitors, $^{*}P < 0.05$; $^{**}P < 0.01$, Newman–Keuls test.

Finally, the tachykinin $NK_2$ receptor agonist, [Ala$^5$,β-Ala$^8$]α-neurokinin-(4-10) did not produce a nociceptive response, even at the high doses studied (up to 20 μg/rat; data not shown). No higher doses were assayed because the injection of 20 μg caused motor effects consisting of reversible paresia of the hindquarters. As no nociceptive effects were obtained, the drug was excluded from further studies.

### 3.2. Effects of i.t. injection of W-7 and calmidazolium on the nociceptive behavior induced by i.t. NMDA (4 μg)

Injection of the calmodulin inhibitors, W-7 (0.25–1 μmol/rat) or calmidazolium (0.12–0.5 μmol/rat), together with 4 μg of NMDA, reduced the nociceptive behavior in a dose-dependent way (Fig. 2). In all cases inhibition at each dose was least during the first 5 min and increased with time. Thus, 0.25 μmol of W-7 and 0.12 μmol of calmidazolium did not modify the effect induced by NMDA during the first 5 min but strongly reduced it 10 and 15 min after injection. The $ED_{50}$ values of calmidazolium and W-7 were 0.17 (0.12–0.23) μmol and 0.35 (0.25–0.49) μmol, respectively, showing that calmidazolium was significantly more potent (2.02-fold) than W-7 in this assay.

### 3.3. Effects of i.t. W-7 and calmidazolium on the nociceptive behavior induced by i.t. AMPA (2 μg)

Neither W-7 (0.5 and 1 μmol/rat) nor calmidazolium (0.25 and 0.5 μmol/rat) antagonized the nociceptive behavior induced by the i.t. injection of AMPA (2 μg). As

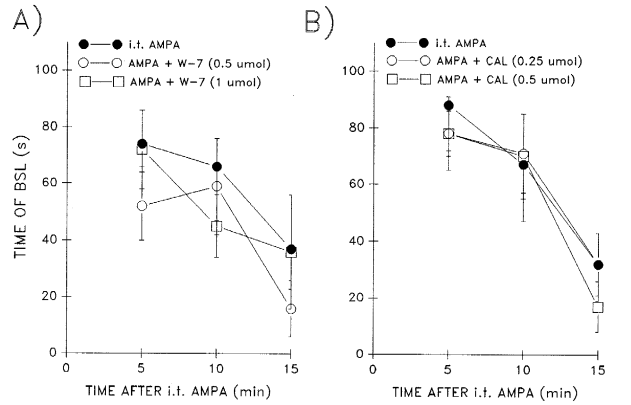


Fig. 3. Effect of i.t. W-7 (0.5 and 1 μmol/rat) (A) and calmidazolium (CAL, 0.25 and 0.5 μmol/rat) (B) on the nociceptive behavior induced by 2 μg of i.t. AMPA. Means and corresponding standard errors are given for three 5-min periods after the intrathecal injection. Comparisons were made for each time between the effect of AMPA alone and in the presence of calmodulin inhibitors.

seen in Fig. 3, the relatively high doses of the two drugs injected did not induce any significant modification of the pain behavior at the times studied.

### *3.4. Effects of i.t. W-7 and calmidazolium on the nociceptive behavior induced by i.t. septide (2 μg)*

W-7 (0.12–0.5 μmol/rat) and calmidazolium (0.06–0.25 μmol/rat) dose dependently inhibited the nociceptive behavior in response to the i.t. injection of septide (2 μg) (Fig. 4). Their inhibitory effect increased with time because 0.12 μmol of W-7 and 0.06 μmol of calmidazolium did not affect the nociceptive response measured during the first 5 min but were effective during the remaining period studied. The $ED_{50}$ showed that calmidazolium ($ED_{50}$ = 0.07 μmol; 0.045–0.12 μmol) was significantly more potent than W-7 ($ED_{50}$ = 0.24 μmol; 0.11–0.51 μmol).

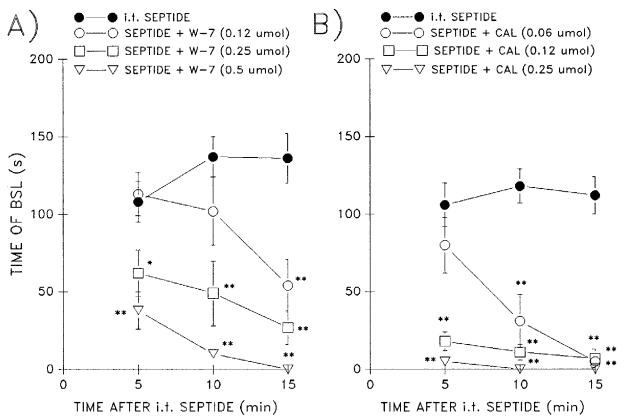


Fig. 4. Effect of i.t. W-7 (0.12–0.5 μmol/rat) (A) and calmidazolium (CAL, 0.06–0.25 μmol/rat) (B) on the nociceptive behavior induced by 2 μg of i.t. septide. Means and corresponding standard errors are given for three 5-min periods after the intrathecal injection. Comparisons were made for each time between the effect of septide alone and in the presence of calmodulin inhibitors, $^{*}$ $P < 0.05$; $^{**}$ $P < 0.01$, Newman–Keuls test.

## 4. Discussion

As previously described, the i.t. injection of molecules involved in nociceptive transmission, or their synthetic analogs, induces a 'nociceptive-related' behavior localized to the regions whose sensitive afferents converge into the injected region of the spinal cord. The reaction consists of biting, scratching and/or licking of the corresponding dermatomes. The fact that analgesic drugs can inhibit it, supports the notion that nociceptive integration is responsible for this behavior (Aanonsen and Wilcox, 1987).

Our data are consistent with previous reports that i.t. injection of AMPA, NMDA and septide are able to induce nociceptive (biting, scratching and licking) behavior in both rats and mice (Hylden and Wilcox, 1981; Aanonsen and Wilcox, 1987; Papir-Kricheli et al., 1987; Björkman et al., 1994). However, the tachykinin $NK_2$ receptor agonist, [$Ala^5$,β-$Ala^8$]α-neurokinin-(4-10), fails to induce a similar reaction. As mentioned in Section 1, although tachykinin $NK_2$ receptors have been implicated in spinal hyperalgesia (Xu et al., 1991; Lepre et al., 1994), direct nociceptive effects have only been described in mice (Gamse and Saria, 1986; Ravard et al., 1994).

Calmidazolium (Gietzen et al., 1981) and W-7 (Tanaka et al., 1982) are among the most specific and selective calmodulin inhibitors available, although total selectivity has not yet been achieved. Due to this lack of absolute selectivity, the use of chemically unrelated calmodulin inhibitors (such as W-7 and calmidazolium) can help (Means et al., 1991) to exclude the involvement of unspecific mechanisms (different from calmodulin inhibition) in their effects.

The calmodulin inhibitors, W-7 and calmidazolium, reduce the nociceptive responses evoked by i.t. NMDA in a dose-dependent way. This result can be understood when one considers that the opening of NMDA receptor channels produces an important influx of $Ca^{2+}$ (MacDermott et al., 1986), able to activate neural calmodulin. The inhibition of biting, scratching and licking behavior induced by NMDA increases with time and only the higher doses were effective during the first 5 min. This could have pharmacokinetic reasons, the calmodulin inhibitors needing to cross the cell membrane to act, whereas all the agonists assayed act directly at membrane receptors. Another explanation could be that calmodulin activation comes into play progressively after $Ca^{2+}$ entry so that the effect due to the inhibition of calmodulin would become more evident with time. In this assay, calmidazolium was twice as potent as W-7, as discussed below.

The lack of analgesic effect of W-7 and calmidazolium when coadministered with AMPA could be explained by

the fact that AMPA receptor channels are mainly permeable to $Na^+/K^+$ ions (Collingridge and Lester, 1989). Marked $Ca^{2+}$ mobilization should not be expected after i.t. AMPA injection, considering that only a small percentage of rat AMPA receptors contains the subunit GluR2(Q) that makes AMPA receptors permeable to $Ca^{2+}$ (Seeburg, 1996). Thus, the cellular events activated by AMPA could possibly remain unaffected by calmodulin inhibitors, as occurred in the present study.

Both W-7 and calmidazolium inhibited the septide-produced nociceptive reaction in a dose-dependent manner. Lower doses of both drugs were necessary to inhibit this response than the response induced by NMDA. Septide was initially described as a selective 'typical' tachykinin $NK_1$ receptor agonist (Papir-Kricheli et al., 1987; Sakurada et al., 1991). There are recent reports that this drug could act at a specific site of the tachykinin $NK_1$ receptor, different from that stimulated by substance P in the same receptor (Maggi et al., 1993; Glowinski, 1995), thus explaining that septide is not a good competitor of substance P in binding assays (Torrens et al., 1995). It is however accepted that septide selectively stimulates tachykinin $NK_1$ receptors and, accordingly, can increase $IP_3$ levels, as do tachykinin $NK_1$ 'typical' receptor agonists (Torrens et al., 1995). The immediate consequence of $IP_3$ formation is the release of $Ca^{2+}$ from intracellular stores. Since the subsequent $Ca^{2+}$ rise might be sufficient to activate calmodulin (Rasmussen et al., 1990; MacNicol and Schulman, 1992), this could help to explain the effects of W-7 and calmidazolium on septide-induced behavior. Additionally, the high potency of W-7 and calmidazolium for inhibition of the nociceptive reaction evoked by septide could also derive from the recently described inhibition by calmodulin inhibitors of the increase of $IP_3$ subsequent to the stimulus of a receptor coupled to the phosphoinositide breakdown pathway (Larsson and Alling, 1995). As with NMDA, calmidazolium was more potent than W-7 to inhibit septide-induced nociceptive behavior. The higher potency of calmidazolium as analgesic, against either NMDA- or septide-evoked nociceptive effects, is consistent with its higher affinity for binding to calmodulin in 'in vitro' assays (Johnson and Mills, 1986).

These results seem to indicate that calmodulin inhibitors can discriminate among different spinal stimuli and do not produce a non-specific neural depression leading to analgesia, similar to that of local anesthetics. We also showed that calmodulin inhibitors act as analgesics affecting mostly $Ca^{2+}$-related nociceptive transmission (NMDA and septide) and not the $Na^+/K^+$ related one (AMPA). These results further characterize the analgesic effect of these drugs that we studied earlier using the formalin test (Menéndez et al., 1996; Menéndez and Baamonde, 1996). In this latter test, based on nociceptive peripheral stimulation, a presynaptic effect could account for the spinal analgesia the drugs induce since calmodulin inhibitors have been reported to block the release of several neurotransmitters (De Lorenzo et al., 1979). It must be emphasized that the stimuli used in the present experiments do not necessarily involve any neurotransmitter release from the primary afferent neuron, excluding the possibility of a presynaptic effect. Thus, independently of a putative presynaptic effect, the present results seem to confirm that W-7 and calmidazolium can act as analgesics modulating calmodulin activity at the postsynaptic level.

Finally, we showed that the analgesic profile of these drugs is not restricted to the neural excitation triggered by one specific type of membrane receptor since they seem to be able to antagonize the neural excitation in which calmodulin is involved, irrespective of which receptor triggers it (for example NMDA and tachykinin $NK_1$ receptors activation). This simultaneous blockade of the transduction mechanisms of at least two different receptors involved in nociceptive transmission confirms calmodulin inhibition as an interesting new tool for the design of strategies for pain study and management.

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