A novel animal model for testing the in vivo potency of putative local anesthetic compounds

Ph.D. Thesis

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Publications

Full papers related to the Thesis

I. **Safrany-Fark A.**, Petrovszki Z., Kekesi G., Liszli P., Benedek G., Keresztes C., Horvath G. In vivo potency of different ligands on voltage-gated sodium channels. Eur J Pharmacol. 2015, 762:158-64.

II. Tuboly G., Tar L., Bohar Z., **Safrany-Fark A.**, Petrovszki Z., Kekesi G., Vecsei L., Pardutz A., Horvath G. The inimitable kynurenic acid: the roles of different ionotropic receptors in the action of kynurenic acid at a spinal level. Brain Res Bull. 2015, 112:52-60.

Full papers not involved in the Thesis

Safrany-Fark A., Petrovszki Z., Kekesi G., Keresztes C., Benedek G., Horvath G. Telemetry monitoring for non-invasive assessment of changes in core temperature after spinal drug administration in freely moving rats. J Pharmacol Toxicol Methods. 2015, 72:19-25.

Abstracts

Safrany-Fark A., Petrovszki Z., Kekesi G., Liszli P., Benedek G., Nagy K., Horvath G. Motor nerve sensitivity changes caused by N-arachidonoyl-dopamine and capsaicin in rats. XIVth Conference of the Hungarian Neuroscience Society, Budapest, January 17-19, 2013

Horvath G., **Safrany-Fark A.**, Petrovszki Z., Kekesi G., Benedek G. Thermoregulatory and motor disturbances after intrathecal injections in freely moving rats. IBRO International Workshop, Szeged, January 16-17, 2014

Pinke I., **Safrany-Fark A.**, Daubner B., Segatto E. Detection of positional disorders of maxillary first molars in cleft palate patients. 8th International Orthodontic Congress, WFO London, September 27-30, 2015

Safrany-Fark A., Segatto E., Voros L., Pinke I. Analysis of transversal and sagittal dental cast measurements in cleft and control patients. 8th International Orthodontic Congress, WFO London, September 27-30, 2015

Safrany-Fark A., Harsanyi R., Kenyeres K., Pinke I. Determination of survival rate of primary molars in control population, and assessment of the dental age for cleft and control groups. 8th International Orthodontic Congress, WFO London, September 27-30, 2015

Introduction

The nociceptive system is functioning as a warning system; therefore, it has a threshold that is low enough for it to be activated before actual damage has occurred. Analgesic technologies and current postoperative pain management are primarily based on nonsteroidal anti-inflammatory drugs, opioids or local anesthetics. Thus, they can be administrated systemically for central medication, or regionally (epidural, topical and infiltrative analgesia) for local anesthesia. The use of local anesthesia during surgical procedures became prominent during the past decade. Acute pain, accompanying dental manipulations, can also be avoided with the application of these drugs. Beside the pain relief, these drugs have a considerable effect on the motor function as well. The action mechanism of the local anesthetics is the inhibition of the axonal activities by the reduction or total blockade of action potentials. The primary sites of their actions are the voltage gated sodium channels (VGSCs), which are transmembrane proteins essential for the influx of sodium ions that subserve impulse generation and propagation in nerve and muscle cells.

In the peripheral nervous system wide variety of axons can be found. These axons have different physiological properties, function and possible drug targeting sites. Biophysical and functional differences between these axons might have some impact on their inhibitory capabilities, too. Some types of peripheral nerves contain several ligand binding sites along the axonal region (e.g. interleukin-6 receptor, γ-aminobutyric acid receptor, protease-activated receptor 1, transient receptor potential vanilloid 1 (TRPV1) and insulin receptors) providing a way to influence their function. These receptors are present primarily on C-type sensory axons and less expressed on motor nerves. Therefore, motor nerves might be more predictable model for the investigation of the effects of different ligands acting on voltage gated channels.

A wide variety of ligands with diverse chemical properties can have significant effect on VGSC function:

Local anesthetics

The local anesthetics in clinical use are tertiary amines. Lidocaine, bupivacaine and ropivacaine are the most widely used ones.

The axon types have different susceptibility to local anesthetics. The block of all sensory, motor and autonomic fibers may be acceptable in some settings, such as during surgery, but there are many clinical situations where a selective inhibition of some but not other axons would be desirable. There are recent reports of local anesthetic formulations with varying degrees of sensory selectivity. Block of nociceptors to produce analgesia without a loss of proprioception, motor or autonomic function may help early mobilization of patients following knee or hip joint replacement. Therefore, sensory-selective local anesthesia has long been a key goal in drug development. Strichartz firstly described in 1973 that the hydrophilic quaternary lidocaine derivative lidocaine N-ethyl bromide (QX-314) is incapable of diffusion through the membrane lipid bilayer of myelinated nerve fibers. Subsequently, it was shown that QX-314 could enter into the cytoplasm through activated TRPV1 channels (e.g., induced by capsaicin) leading to the preferential block of VGSCs in nociceptors producing a selective antinociception. Furthermore, QX-314 seems to be able to interact with local anesthetics enhancing their effect on sensory and motor functions applied to the mixed, sciatic nerve. However, in mixed nerves the motor responses can be influenced by actions on sensory fibers, at least indirectly, through reflex arches.

Nisoxetine

Nisoxetine is a drug used in the therapy of affective disorders as a potent norepinephrine reuptake inhibitor. The structure of nisoxetine and local anesthetics share important moieties, namely, a lipophilic structure at one end and an amine at the other, thus, it is not entirely unexpected that nisoxetine blocks VGSCs. Thus a recent study found that nisoxetine has a local anesthetic effect after infiltrative cutaneous administration that might be related to its VGSC blocking potency.

Capsaicin, arachidonic acid and arachidonoyl ethanolamide

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) is a well-known vanilloid substance. It is the pungent agent in hot chili peppers, and perhaps one of the most enigmatic molecules ever produced by plants. Capsaicin causes its effects through actions on transient receptor potential cation channel subfamily V member 1 (TRPV1). Some *in vitro* data proved that capsaicin can also influence the activity of VGSCs. Other lipid soluble ligands, polyunsaturated fatty acids e.g. arachidonic acid (AA), and their derivatives also may have significant effect on VGSCs. Anandamide (arachidonoil-etanolamide: AEA), an AA derivative, is an endogenous ligand of cannabinoid (CB) and TRPV1 receptors. Some *in vitro* data showed that both AA and AEA can inhibit the VGSCs' activity, but *in vivo* data are not available in this respect.

Kynurenic acid

Degradation of the essential amino acid, tryptophan, along the kynurenine pathway yields several neuroactive intermediates (kynurenines) including kynurenic acid (KYNA). Kynurenines participate in immunoregulation, inflammation and possess pro- or anti-excitotoxic properties, also their involvement has been suggested in oxidative stress, Alzheimer's, Parkinson's and Huntington's diseases, amyotrophic lateral sclerosis and multiple sclerosis.

Earlier data showed that intrathecal (IT) administration of KYNA caused antinociception accompanied with a reversible, dose-dependent motor impairment similarly to the local anesthetics. Since its effect on VGSC has not yet been investigated, therefore, it cannot be excluded that these effects were produced, at least partially, by VGSC blockade.

Goals

1. The first aim of this study was to evolve an *in vivo* model, appropriate for examining VGSC-mediated effects on nerves containing only motor fibers, and for testing different drugs with different chemical properties.
2. The second goal was to test the reliability of the model by testing the efficacy of three classical local anesthetics (lidocaine, bupivacaine, ropivacaine) on motor nerve function.
3. The third goal was to investigate the *in vivo* potency of the permanently charged sodium channel blocker QX-314 on motor fibers. The possible drug interactions of the combination of QX-314 with lidocaine, as a new way for local anesthesia, was also examined.
4. Additionally, the effects of nisoxetine on VGSC and its conduction blocking efficacy in our animal model were also investigated.
5. The next aim was to provide *in vivo* results regarding the VGSC blocking capability of capsaicin, AA and AEA.
6. Since intrathecally administrated KYNA produced effects similar to classical local anesthetics', our last goal was to provide information about its potency on VGSC.

Methodological background

As it was discussed above, the motor nerves have primarily VGSCs, while sensory fibers have several other ligand binding sites as well, which can influence the excitability of these neurons. Therefore, models containing only motor fibers may provide a simple way for the *in vivo* modulation of the voltage gated channels involved in the action potential, especially VGSCs. Marginal mandibular branch of the

facial nerve in rats, that controls the muscle activity of the vibrissae, contains only motor fibers. Even muscle spindles are lacking in vibrissae muscles, therefore, it can be an appropriate model for moderately selective influence of these channels. Whisker excursion, or "whisking," is the most readily measurable facial movement in the rat. This model was chosen for our electrophysiological study, because the mandibular branch is ideal for the surgical protocol. Electrodiagnostic studies, originated in the 19th century, are consistently used over the past 30–40 years. The most common tests are nerve conduction studies (NCS) and electromyography (EMG).

Methods

Animals, drugs and experimental setup

Wistar rats were anesthetized with an intraperitoneally injected mixture of ketamine (72 mg/kg) and xylazine (8 mg/kg). The facial area of the anesthetized animals was shaved, an "L"-shape incision was made and the skin was gently elevated to expose the marginal mandibular branch of the facial nerve at buccal level. The proximal part was wrapped with a unipolar wire electrode (0.1 mm) for electrical stimulation to induce the whisker movement. Unipolar needle (26-gauge) electrodes were placed into the whisker area of the rats to investigate muscle activity. The ground electrode was placed subcutaneously, close to the nerve and the muscle. The stimulations with rectangular biphasic pulses of constant current were delivered through a stimulator with a supramaximal stimulus (1 mA for 250 μs), and EMG activities after repeated single stimuli were recorded. After determinations of baseline activities, the effects of the different ligands were investigated. Drugs were applied perineurally distal to the stimulating electrode in 20 μL volume.

The number of the animals in the different groups were between 6-10. The measurements were repeated 30, 60, 90, 120 s after the drug administration, and then in 2 minute intervals for 30 min in total. The mean values between 0.5–2, 4–10, 12–20 and 22–30 min intervals were analyzed as 1^{st}, 2^{nd}, 3^{rd} and 4^{th} time periods.

Amplitudes were normalized by calculating the percentage change from baseline for each post-injection data point.

The area under the curve (AUC) values were obtained by calculating the area during the 30 min period following the injection to construct dose response curves for the different ligands. The time-course

effects were examined by repeated measurement of ANOVA. The post-hoc comparison was calculated by using the Newmann-Keuls test (p value <0.05 was considered significant).

Results

Classical local anesthetics

All of these drugs (lidocaine, bupivacaine and ropivacaine) produced prolonged EMG depression in higher doses. The linear regression curves of AUC data showed dose dependent effects of all ligands with slight potency differences among them; thus, the ED_{50} value was the highest for lidocaine, while bupivacaine and ropivacaine had slightly higher potency. Regarding their effects at the 1st period lidocaine was more effective than the other two drugs, indicating its faster effect. However, the effect of lidocaine slightly decreased during the 4th period, while ropivacaine and bupivacaine resulted in a prolonged anesthesia. Regarding the application of QX-314 (380 nmol), it did not produce any effect by itself, but prolonged the effects of lidocaine in their combinations.

Nisoxetine

The linear regression curve of nisoxetine revealed that nisoxetine had very low potency compared to the classical local anesthetic drugs.

Capsaicin, arachidonic acid and arachidonoyl ethanolamide

As regards the capsaicin treatment, it produced about 50% inhibition in a high dose (663 nmol). ANOVA showed significant effects of treatment after AEA application; thus, 567 nmol produced about 50% inhibition, but the post-hoc analysis did not show significant differences between the vehicle and AEA at any time points. Regarding the effects of AA, it did not produce significant inhibition on the evoked action potentials even in high doses.

Kynurenic acid

The perineural administration of high dose KYNA (528.6 nmol, 100 μg) that resulted motor impairment and antinociception at the spinal level, did not influence the evoked responses.

Discussion

The present results revealed that this *in vivo* model can provide reliable data, for at least half an hour, about the motor nerve excitability, since the responses to repeated stimuli did not change during this period, and none of the vehicles caused changes in the stimulus-response curves.

We showed that classical local anesthetics inhibited the evoked action potentials with high potency in a nerve containing motor fibers exclusively. Slight potency differences were found between these ligands. Furthermore, it was revealed that QX-314 did not produce any effects on motor fiber function by itself, but it slightly prolonged the effect of lidocaine in agreement with the earlier behavioral studies, which described a potentiated motor paralysis after perisciatic co-administrations of QX-314 with bupivacaine or lidocaine.

Our results revealed that nisoxetine had a low potency in this model. Some data show that nisoxetine inhibits VGSCs *in vitro* models, and also produces dose dependent blockade during spinal anesthesia in rats. The controversy might be due to the differences in the applied model as the earlier study investigated only the sensory functions, and the multiple differences between the motor and sensory fibers could have led to decreased potency in motor fibers.

Our data firstly revealed the *in vivo* effects of the polyunsaturated fatty acid (AA), its derivative (AEA) and capsaicin on EMG activity, showing that AA was ineffective, while AEA and capsaicin had a modest blocking effect in high, but not in the maximally applied dose. Thus, in contrast to the *in vitro* results, our data suggest that they do not have significant effects on VGSCs in moderate doses.

IT administration of KYNA produces antinociception in different pain models, but its effective doses also caused motor impairment similarly to the classical local anesthetics. As KYNA had no effects at high (100 μg) dose on the motor nerve function, therefore, we suggest that the anesthetic effects of KYNA at the spinal level might not be due to the inhibition of VGSCs, but do the receptor antagonist effect at the NMDA receptors.

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