

# The Pentapeptide QYNAD Does Not Inhibit Neuronal Network Activity

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**ABSTRACT:** *Background:* Controversial data was published about the sodium channel-blocking effect of the endogenous pentapeptide QYNAD, which is elevated in patients with multiple sclerosis and Guillain-Barré-syndrome. In some experiments with single cells and nerve preparations QYNAD inhibited sodium currents to the same extent as the known sodium channel blocker lidocaine whereas in other laboratory testing QYNAD failed to show any effect at all. *Methods:* Micro-electrode arrays with cultured neuronal networks are highly suitable to determine neuroactive activity of applied substances. The impact on electrophysiological parameter changes was compared between QYNAD and the established sodium channel blockers lidocaine and tetrodotoxin (TTX). *Results:* QYNAD did not alter network activity whereas the sodium channel blockers lidocaine (IC<sub>50</sub> 14.9 μM) and tetrodotoxin (IC<sub>50</sub> 1.1 nM) reversibly decreased network activity in similar concentrations as in patch-clamp experiments. This decrease of spontaneous electrophysiological activity was achieved by prolonging the interburst-interval. *Conclusion:* Although QYNAD might have mild effects on single-cell sodium currents, there is no significant effect on neuronal network function. These results raise concerns about QYNAD exhibiting a relevant impact on functional disability of the central nervous system in patients.

**RÉSUMÉ:** *Le pentapeptide QYNAD n'inhibe pas l'activité d'un réseau neuronal.* *Introduction:* Les données publiées sur le blocage des canaux sodiques par le pentapeptide QYNAD, un peptide endogène, sont controversées. Le taux de ce pentapeptide est élevé chez les patients atteints de sclérose en plaques et dans le syndrome de Guillain-Barré. Dans certaines expériences sur des préparations de cellules et de nerfs isolés, le QYNAD inhibe les courants sodiques de façon aussi importante que la lidocaïne, un inhibiteur des canaux sodiques, alors que dans d'autres expériences, il n'a aucun effet. *Méthodes:* Les matrices d'électrodes sont très appropriées pour déterminer la neuroactivité de substances appliquées à des réseaux neuronaux en culture. Nous avons comparé l'impact du QYNAD et de la lidocaïne, un bloquant des canaux sodiques bien connu, et de la tétrodotoxine (TTX) sur les changements de paramètres électrophysiologiques. *Résultats:* Le QYNAD ne modifiait pas l'activité du réseau neuronal alors que la lidocaïne, un bloquant des canaux sodiques (IC<sub>50</sub> de 14,9 mmol) et la TTX (IC<sub>50</sub> de 1,1 nmol) diminuaient de façon réversible l'activité du réseau neuronal à des concentrations semblables à celles utilisées dans les techniques « patch-clamp ». Cette diminution de l'activité électrophysiologique spontanée était obtenue en prolongeant l'intervalle entre les décharges. *Conclusion:* Bien que le QYNAD puisse avoir de légers effets sur les courants sodiques dans un système unicellulaire, il n'a pas d'effet significatif sur la fonction d'un réseau neuronal. Ces résultats soulèvent des doutes quant à l'impact du QYNAD sur l'invalidité fonctionnelle du système nerveux central chez des patients.

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Multiple sclerosis (MS) is one of the most common chronic inflammatory neurological diseases, characterized by demyelination and axonal damage.<sup>1</sup> Rapid clinical fluctuations in patients may be caused by soluble factors detectable within the cerebrospinal fluid (CSF). Cerebrospinal fluid of these patients (MS-CSF) was found to decrease sodium currents in human myoballs and rat cortical neurons and to induce axonal damage.<sup>2-4</sup> In peripheral immune-mediated diseases like Guillain-Barré-syndrome (GBS), sera from patients induced myelin disintegration and conduction block in cell cultures.<sup>5</sup> The endogenous pentapeptide QYNAD (Gln-Tyr-Asn-Ala-Asp),

which is measured at elevated levels in the CSF and serum of patients with MS and GBS, has been reported to account for the inhibition of sodium currents.<sup>6</sup> QYNAD, isolated from MS-CSF as well as the synthesized peptide, reduced sodium currents in

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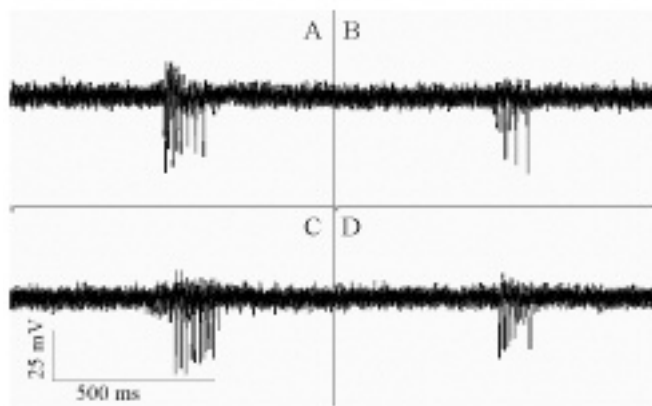
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human neuroblastoma cells at a concentration of about 10  $\mu\text{M}$ . This blocking effect was similar to lidocaine in a concentration of 50  $\mu\text{M}$ <sup>6</sup> and has also been reported in acutely isolated thalamic neurons with a maximum effect at 10  $\mu\text{M}$ .<sup>7</sup> However, other laboratories using synthesized QYNAD batches from multiple producers could not confirm this blocking effect, neither for recombinant nor native voltage-gated sodium channels in a variety of cells and isolated nerves.<sup>8,9</sup> As the central nervous system in vivo is composed of tightly connected neuronal networks, the aim of this study was to test the impact of synthesized QYNAD on neuronal network activity and to compare the results with the effects of the established sodium channel-blockers lidocaine and tetrodotoxin (TTX). In our experiments we used the novel multielectrode array technique because spontaneously active neuronal networks on these arrays are highly sensitive biosensors for the integrative function of axonal, dendritic and synaptic function.<sup>10</sup>

## METHODS

Cryopreserved embryonic rat cortical neurons (QBM Cell Science, Ottawa, Canada) were diluted with prewarmed B27-supplemented neurobasal medium (Invitrogen, Karlsruhe, Germany) and plated onto poly-D-lysine- and laminin-coated microelectrode arrays (multielectrode array's (MEAs); Multi Channel Systems, Reutlingen, Germany) in a density from 1.5 to 4  $\times 10^5/\text{cm}^2$ . Extracellular potentials were recorded on (MEAs with a square grid of 60 planar Ti/TiN-microelectrodes (30  $\mu\text{m}$  diameter, 200  $\mu\text{m}$  spacing) with an input impedance of <50 k $\Omega$



**Figure 1:** Extracellular field potentials recorded simultaneously from four electrodes. Distance between electrodes A-B 1.3 mm, C-D 894  $\mu\text{m}$ , A-C 894  $\mu\text{m}$ , B-D 1.28 mm, A-D 566  $\mu\text{m}$  and B-C 447  $\mu\text{m}$ .

according to the specifications of the manufacturer (Figure 1). Signals from all 60 electrodes were simultaneously sampled at 25 kHz, visualized and stored using the standard software MCRack. Individually for each channel, the threshold for spike detection was set to eight standard deviations of the average noise amplitude during a 10% “learning phase” at the beginning of each measurement. An absolute refractory period of 4 ms and

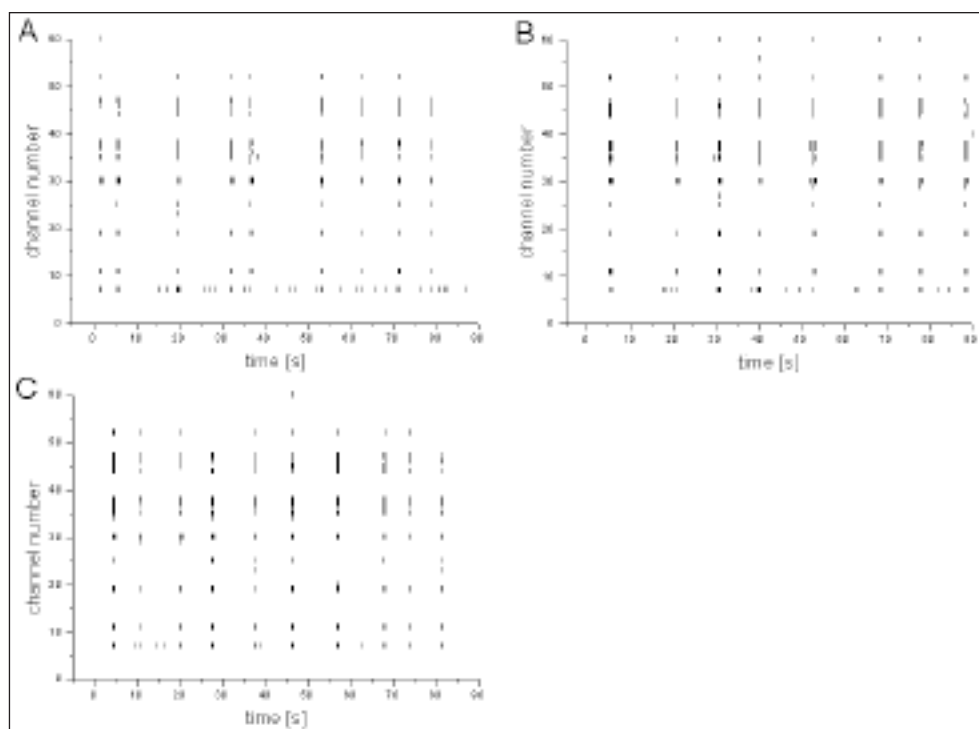
a maximum spike width of 2 ms were imposed on the spike detection algorithm. All spike waveforms were stored separately and visually inspected for artifacts. The burst detection relied on an entropy based algorithm. A minimum entropy of five was required for a sequence of at least three spikes to constitute a burst. Inter-burst intervals (IBIs) were determined as time differences between the first spikes of subsequent bursts. Bursts were also checked by visual inspection for plausibility. Recordings of spontaneous spike activity were performed between 19 and 116 days in vitro (DIV). Analysis of the network parameters spike rate, burst rate, burst duration, inter-burst interval and number of spikes per burst were performed offline by custom-built software (Result, Tönisvorst, Germany). Every measurement comprised three recordings – control, test substance (QYNAD, lidocaine or TTX) and second control – each three minutes long and separated by an intermediate delay of 60 seconds. Recordings were made in magnesium-free bath solution [NaCl 150 mM, KCl 4 mM, CaCl<sub>2</sub> 2 mM, HEPES 10 mM, Glucose 10 mM, pH 7.4] as well as in artificial CSF [ACSF; in mmol/l: NaCl 150, KCl 3, CaCl<sub>2</sub> 1, MgCl<sub>2</sub> 1, HEPES 10, Glucose 10, pH 7.4]. To test for possible impacts of HEPES on QYNAD efficacy, one QYNAD batch was also dissolved in bicarbonate-buffered ACSF [in mmol/l: NaCl 128, KCl 3, CaCl<sub>2</sub> 1, MgCl<sub>2</sub> 1, NaHCO<sub>3</sub> 22, NaH<sub>2</sub>PO<sub>4</sub> 1.25, Glucose 10, pH 7.4 adjusted by continuous CO<sub>2</sub> flow 5%]. Recordings were made at 37°C (head stage heating). PH-value was stable between 7.3 and 7.4 in all experiments.

QYNAD batches were synthesized by ThermoElectronGmbH (Ulm, Germany), by the Biologisches-Medizinisches Forschungszentrum of the University of Düsseldorf (Germany) and by the Institut für Zellbiologie (kindly provided by Dr. Wiendl, Department of Neurology, Tübingen, Germany). Data are presented as mean  $\pm$  standard deviation, n refers to the number of recordings. Dose response curves with IC<sub>50</sub> values were fitted to the data according to the Hill equation using nonlinear least squares regression (ORIGIN 5.0, Microcal Software, Northampton, USA).

## RESULTS

### I. QYNAD

Five different batches of QYNAD were used in this study, one of them being acetylated. In concentrations between 100 nM and 800  $\mu\text{M}$  (n= 50) QYNAD did not inhibit neuronal network activity, neither in bath solution nor in HEPES-buffered artificial cerebral spinal fluid (ACSF) or in bicarbonate-buffered ACSF (Figure 2a). After application of 10  $\mu\text{mol/l}$  QYNAD the mean network activity was 88.8 %  $\pm$  17.1% of control value. Comparison of network parameters in eight measurements after application of 100  $\mu\text{M}$  QYNAD showed no change in number of spikes per minute (ratio 1.1  $\pm$  0.2), number of bursts per minute (ratio 1.2  $\pm$  0.2), number of spikes per burst (ratio 1.1  $\pm$  0.2), burst duration (ratio 1.0  $\pm$  0.2), duration of inter-burst intervals (ratio 1.1  $\pm$  0.2) and of inter-spike intervals (ratio 1.0  $\pm$  0.1) (Figure 2b). Even prolonged application for 20-25 minutes in concentrations between 10  $\mu\text{M}$  and 300  $\mu\text{M}$  (n=6) had no effect on spontaneous spike activity (Figure 3).



**Figure 2a:** Spike raster plots of spontaneously active cryopreserved cortical neurons on one MEA in HEPES-buffered ACSF. Each single bar represents one spike; the channel numbers are displayed on the vertical axis. The network exhibits correlated burst activity (thick bars) across several electrodes. A) ACSF B) Acetylated QYNAD [100  $\mu$ M] 1 minute after application C) wash-out with ACSF.

## II. TETRODOTOXIN

Tetrodotoxin (TTX) (100 pM – 100 nM, dissolved in bath solution; n=35) reversibly inhibited neuronal network activity in a dose-dependent manner. A formal fit of the inhibition of spontaneous spike rate to the Hill equation resulted in a dose response curve with an  $IC_{50}$  of  $1.1 \text{ nM} \pm 0.2 \text{ nM}$  (Figure 4). This inhibition was caused by an extension of the inter-burst interval.

## III. Lidocaine

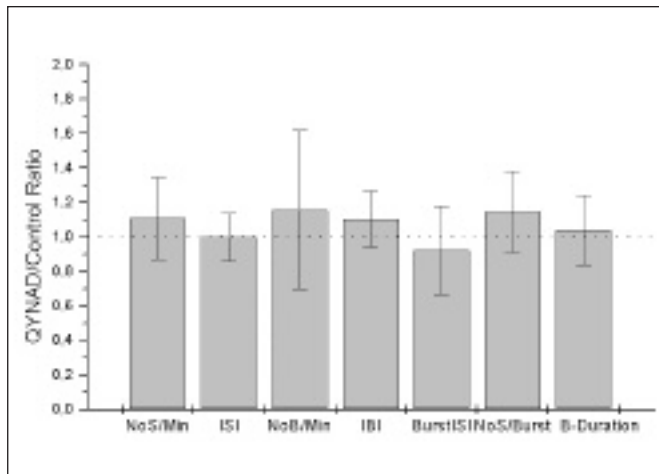
Lidocaine (diluted in ACSF) decreased neuronal network activity in a dose dependent manner (100 nM – 1mM; n=24; Figure 4). One mM lidocaine blocked spontaneous spike rate (SSR) completely. A formal fit to the data resulted in an  $IC_{50}$  value of approximately  $14.9 \text{ } \mu\text{M} \pm 2.7 \text{ } \mu\text{M}$ . Following application of 50  $\mu\text{mol/l}$  lidocaine the network activity was reduced to  $13.3\% \pm 15.4\%$  of control value. The inhibition resulted from an increase of the inter-burst interval.

## DISCUSSION

Microelectrode arrays with neuronal networks allow convenient monitoring of spontaneous electrical activity of excitable cells and can be used as cell-based biosensors for neuroactive substances.<sup>10</sup> The cryopreserved rat cortical neurons used in this study have been shown to exhibit the same electrophysiological properties as freshly prepared cortical neurons of the rat.<sup>11</sup> In the present study, TTX ( $IC_{50}$  1.1 nM) and

lidocaine ( $IC_{50}$  14.9  $\mu\text{M}$ ) remarkably reduced network activity in similar concentrations as in patch-clamp experiments, pointing to the high sensitivity of this biosensor. For QYNAD, we found no acute inhibitory effect on rat cortical networks on MEAs in a concentration range from 100 nM to 800  $\mu\text{M}$ . This concentration range was selected in order to comprise the relevant concentrations measured in patients. QYNAD concentrations measured in CSF are below 0.16  $\mu\text{M}$  in healthy individuals, 11–34  $\mu\text{M}$  in MS-patients, 25–323  $\mu\text{M}$  in patients with GBS and in serum of GBS-patients 10–164  $\mu\text{M}$ .<sup>12,13</sup> We have no evidence that QYNAD (acetylated and non-acetylated) affects the neuronal network in the same way as TTX or lidocaine. In particular, 10  $\mu\text{M}$  QYNAD did not inhibit the network activity to the same extent as 50  $\mu\text{M}$  lidocaine as described in single cell patch-clamp recordings.<sup>6</sup>

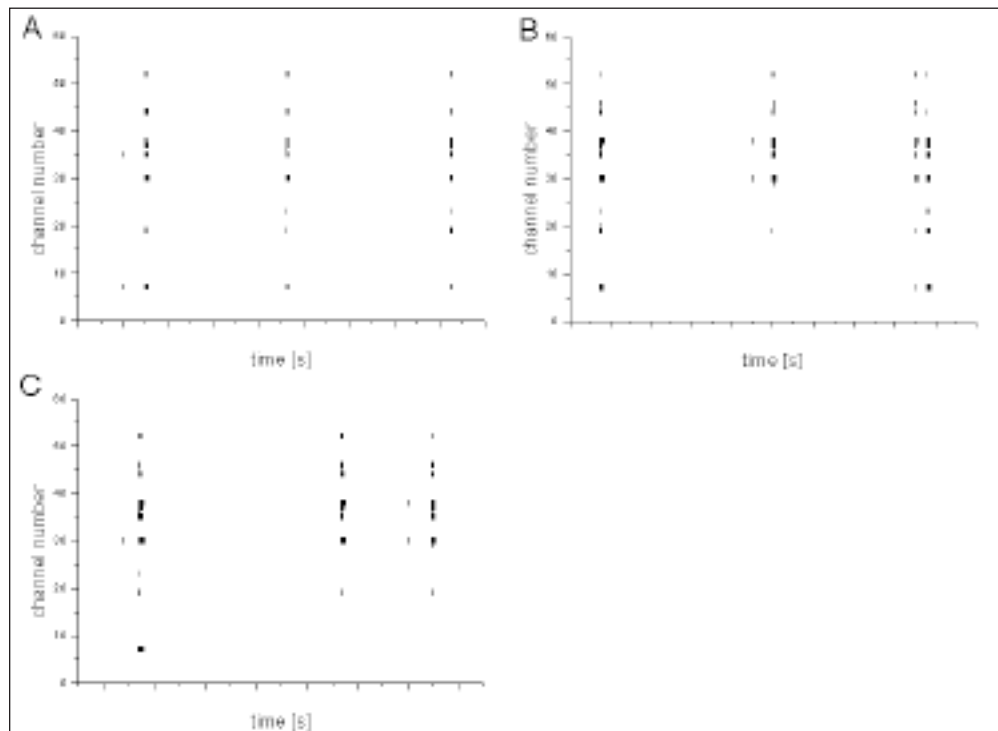
The electrophysiological experiments with QYNAD published so far differ in a) investigated sodium channel isoforms, b) duration of equilibration in bath solution before recording and c) manufacturer of QYNAD batches. As QYNAD used in this study was prepared by three different manufacturers it is unlikely that the ineffectiveness was due to insufficient synthesis. Acetylation of the N-terminal amino group should prevent formation of the less effective pyroglutamic acid derivative, pyQYNAD. The batch of acetylated QYNAD also failed to inhibit neuronal network activity in our experiments. QYNAD was dissolved in recording solution at least 15 minutes prior to experiment and equilibration in the recording chamber was allowed for one



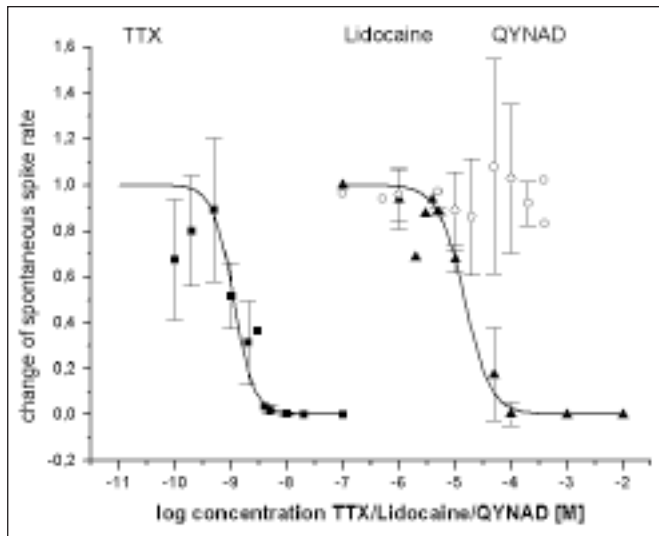
**Figure 2b:** Electrophysiological parameters measured are number of spikes per minute (No S/minute), inter-spike-interval (ISI), number of bursts per minute (No B/minute), inter-burst-interval (IBI), intra-Burst-ISI (Burst ISI), number of spikes per burst (No Sp/burst) and the burst duration (B-duration). After application of 100  $\mu\text{M}$  QYNAD ( $n = 8$ ) no significant change of these network parameters expressed as the QYNAD/control ratio was observed. Data are presented as mean  $\pm$  standard deviation.

minute before recording. Padmashri et al.<sup>14</sup> stated that an equilibration duration of 15 minutes was crucial for proper blocking activity of QYNAD. In contrast, the effect occurred within a few seconds in the original report of Brinkmeier<sup>6</sup> and in our experiments even after a recording time of 20-25 minutes QYNAD in concentration of 10  $\mu\text{M}$ , 100  $\mu\text{M}$  and 300  $\mu\text{M}$  failed to show any inhibiting effect. Some authors suggested a sodium-channel subtype-specific effect of QYNAD on  $\text{Na}_v1.2$  channels.<sup>7,14</sup> We did not specifically look for  $\text{Na}_v1.2$  in our experimental setup (rat embryonic neurons prepared at day E18/E19), but  $\text{Na}_v1.2$  is generally present in embryonic rat brain at stages E15 to E19.<sup>15</sup> Cryopreservation had no electrophysiological influence on spike activity mainly driven by sodium channel action potentials. Even the  $\text{IC}_{50}$  for TTX sensitivity was comparable in cryopreserved and freshly dissociated cortical neurons (Otto et al., 2004). Therefore, we have no evidence that cryopreservation might substantially decrease  $\text{Na}_v1.2$  channels.

The recording temperature generally influences the channel function and peptide kinetics. In experiments showing a blocking effect of QYNAD, recording temperature was between 15°C and 32°C. In one report a reduced effect of QYNAD was observed at 32°C compared to room temperature which was thought to be due to formation of pyroglutamic acid derivative.<sup>7</sup> In experiments showing no blocking effect of QYNAD, recording temperature was between 19°C and room temperature for single cells and 37°C for optic and sciatic nerve. In order to mimic physiological



**Figure 3:** Spike raster plots of spontaneously active cryopreserved cortical neurons on one MEA in HEPES-buffered ACSF. Each single bar represents one spike; the channel numbers are displayed on the vertical axis, time period of the horizontal axis is 20 seconds. A) ACSF for 22 minutes B) Acetylated QYNAD [100  $\mu\text{M}$ ] 20 minutes after application C) wash-out with ACSF for 14 minutes.



**Figure 4:** Dose-response diagram comparing the effects of TTX (■,  $n = 35$ ), Lidocaine (▲,  $n = 24$ ) and QYNAD (○,  $n = 50$ ) on spontaneous network activity. A fit adapted to the Hill equation revealed an  $IC_{50}$  of 1.1 nM for TTX and nearly 15  $\mu$ M for Lidocaine, whereas QYNAD had no inhibitory effect in concentrations below 1 mM.

in vivo conditions, our experiments were carried out at 37°C. Concerning the possible pathophysiological relevance of QYNAD, it is important to mention that rapid clinical deterioration in patients does not occur with low temperature but with increased temperature, known as the Uthoff-Effect.

Furthermore, QYNAD failed to show any effect on severity of disease, relapse frequency and persistence of neurological symptoms when applied intraperitoneally experimental autoimmune encephalitis (EAE), the animal model of relapsing and chronic MS.<sup>7</sup> Additionally, QYNAD concentrations did not correlate with clinical progression or magnetic resonance imaging lesions in patients with multiple sclerosis.<sup>16</sup> Although QYNAD might have mild sodium-blocking-effects on single cells as reported, the electric transmission in neuronal networks and in vivo seems not to be affected. Thus, in our opinion it is unlikely that QYNAD contributes to the fast changes of symptoms in patients. QYNAD might not be the relevant blocking factor but rather represent the decomposition of larger proteins like ankyrins<sup>9</sup>, which interact with sodium channels. The controversial data on QYNAD indicates a further challenge to identify neuroactive components in inflammatory mediated diseases like multiple sclerosis.

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