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Efficacy and tolerability of the Galanin Analog NAX 5055 in the multiple-hit rat model of symptomatic infantile spasms

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Abstract

Infantile spasms are seizures manifesting in infantile epileptic encephalopathies that are associated with poor epilepsy and cognitive outcomes. The current therapies are not always effective or are associated with serious side effects. Early cessation of spasms has been proposed to improve long-term outcomes. To identify new therapies for infantile spasms with rapid suppression of spasms, we are using the multiple-hit rat model of infantile spasms, which is a model of refractory infantile spasms. Here, we are testing the efficacy and tolerability of a single dose of the galanin receptor 1 preferring analog, NAX 5055, in the multiple-hit model of spasms. To induce the model, postnatal day 3 (PN3) male Sprague-Dawley rats underwent right intracerebral infusions of doxorubicin and lipopolysaccharide; p-chlorophenylalanine was then injected intraperitoneally (i.p.) at PN5. After the onset of spasms at PN4, 11–14 rats/group were injected i.p. with either NAX 5055 (0.5, 1, 2, or 4 mg/kg) or vehicle. Video monitoring for spasms included a 1hour pre-injection period, followed by 5 hours of recording post-injection, and two 2 hour sessions on PN5. The study was conducted in a randomized, blinded manner. Neurodevelopmental reflexes were assessed daily as well as at 2 hours after injection. Respiratory function, heart rate, pulse distension, oximetry and blood glucose were measured 4 hours after injection. The relative expression of GalR1 and GalR2 mRNA over β-actin in the cerebral cortex and hippocampus was determined with real time reverse transcription polymerase chain reaction. There was no acute effect of NAX 5055 on spasm

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Disclosures

MJG, MK, and ASG have no conflict of interest in the work reported in the present manuscript. HSW is a scientific cofounder and BDK is an employee of NeuroAdjuvants, Inc. Salt Lake City UT. The Albert Einstein College of Medicine holds a patent for the multiple-hit model (# 7863499). No royalties have been received or other financial interests exist on account of this patent. The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving animals http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm.

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frequency after the single dose of NAX 5055 (n=11–13 rats/group, following exclusions). Neurodevelopmental reflexes, vital signs, blood glucose measured 4 hours post-injection, and survival were not affected. A reduction in pulse and breath distention of unclear clinical significance was observed with the 7mg/kg NAX 5055 dose. GalR1 mRNA was present in the cerebral cortex and hippocampus of PN4 and adult rats. The hippocampal –but not the cortical- GalR1 mRNA expression was significantly lower in PN4 pups than in adults. GalR1 mRNA was also at least 20 times less abundant in the PN4 cortex than GalR2 mRNA. In conclusion, a single dose of NAX 5055 has no acute efficacy on spasms or toxicity in the multiple hit rat model of medically refractory infantile spasms. Our findings cannot exclude the possibility that repetitive NAX 5055 administration may show efficacy on spasms. The higher expression of GalR2 in the PN4 cortex suggests that GalR2-preferring analogs may be of interest to test for efficacy on spasms.

Keywords

Antiepileptic; Galanin receptor; glucose; antibody; neurodevelopmental reflexes; cerebral cortex

Introduction

Infantile spasms (IS) occur as clusters of epileptic spasms in the context of epileptic encephalopathies of infancy. IS are often associated with poor outcomes, including intractable epilepsies and cognitive deficits (Pellock et al., 2010; Riikonen, 2001b). IS do not typically respond to the usual antiseizure drugs. The current treatments for IS include adrenocorticotropic hormone (ACTH) and vigabatrin, which are not always effective on spasms, may have serious side effects and may not improve significantly the associated cognitive decline (Darke et al., 2010; Lux et al., 2004; Riikonen, 2001a; 2001b). Unfortunately, only 54 - 87% of patients in various small cohorts of IS patients are free of spasms 14 days after introduction of appropriate treatments (Lux et al., 2004; Mackay et al., 2004; Pellock et al., 2010). IS due to structural/metabolic etiologies are more often drug-resistant (Riikonen, 2010), and cognitive outcome is usually worse than IS of unknown etiology (O'Callaghan et al., 2011). Early cessation of the spasms has been proposed to improve outcome, at least in patients with IS of unknown etiology (Darke et al., 2010; Kivity et al., 2004; Lombroso, 1983; O'Callaghan et al., 2011). New therapies are needed to provide faster and complete control of spasms, with the ultimate goal being the improvement of short and long-term epilepsy and cognitive outcomes.

Several acute and chronic rodent models of IS have been developed recently to elucidate the pathophysiology and facilitate the identification of new, improved therapies for IS (Chudomelova et al., 2010; Galanopoulou, 2013). Here, we are using the multiple-hit chronic rat model of IS which models IS due to a structural underlying lesion (Scantlebury et al., 2010). The multiple-hit model of IS reproduces an early life epileptic encephalopathy with age-specific expression of clusters of epileptic spasms (postnatal day (PN) 4–13), later appearance of other seizure types, and poor neurodevelopmental outcomes. In the multiple-hit model, spasms are refractory to chronic administration of ACTH and transiently sensitive to vigabatrin, which render this a model of drug-resistant IS (Scantlebury et al., 2010). The multiple-hit model of IS has been successfully used for the identification of new rapid-onset (carisbamate, rapamycin) and disease-modifying (pulse rapamycin) therapies (Ono et al., 2011; Raffo et al., 2011).

Galanin is an inhibitory neuropeptide that regulates feeding, reproduction, growth, pain and neuronal development via activation of the galanin receptors GalR1 and GalR2 (Abbott and Pilowsky, 2009; Melander et al., 1986), and inhibits glutamate release in the hippocampus

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(Mazarati et al., 2001; Mazarati et al., 2000). GalR agonists (Bartfai et al., 2004) or allosteric modulators (Lu et al., 2010), or virus-mediated galanin expression (Haberman et al., 2003) decrease seizure susceptibility and can be neuroprotective. Galanin analogs have demonstrated promising antiseizure properties in preclinical studies (Bulaj et al., 2008; White et al., 2009). NAX 5055 is a galanin analog with higher affinity for GalR1 than GalR2 (Bulaj et al., 2008; White et al., 2009) which showed promising antiseizure effects in models of seizures in adults: the Frings audiogenic seizure-susceptible mouse, the mouse corneal kindling model of partial epilepsy, and the 6 Hz corneal stimulation model, a model of drug-resistant epilepsy (White et al., 2009).

In this study, we test whether a single intraperitoneal (i.p.) injection of NAX 5055 given after the onset of spasms decreases acutely the frequency of spasms in the multiple-hit rat model of drug-resistant IS, using dose and time-response experiments. In parallel, we also assess the tolerability of NAX 5055 in developing rats, in regards to respiratory function, survival, daily weights, and glucose regulation, due to the reported effects of galanin on insulin secretion (Ruczynski et al., 2002; Verchere et al., 1992).

Materials and Methods

Animals

This study was done in litters of 10 male offspring of timed pregnant Sprague-Dawley rats (Taconic farms, Inc., Hudson, NY, USA). The day of birth was considered as PN0. Rat pups were kept with their dam in our animal facility at constant temperature (21–23°C) and humidity (40–60%), in a 12 hours dark/12 hours light cycle with access to water and food ad libitum, according to the guidelines of the American Association for the Accreditation of Laboratory Animal Care. All procedures and experiments were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the Albert Einstein College of Medicine.

The multiple-hit model of IS

Induction of the multiple-hit model of IS requires stereotactic infusions of doxorubicin (5μg/2.5μl, right intracerebroventricular injection) and lipopolysaccharide (3μg/1.5μl, right intraparietal injection) at PN3 under isoflurane anesthesia, following the previously used coordinates (Raffo et al., 2011; Scantlebury et al., 2010). Doxorubicin: 2.68mm anterior to lambda; 1.1 mm lateral to sagittal suture; 3.3 mm deep; lipopolysaccharide: 2.55 mm anterior to lambda; 1 mm lateral to sagittal suture; 1.7 mm deep. The skin was closed with Vetbond (3 M, St Paul, MN) and protected with dental acrylic. P-chlorophenylalanine (PCPA) was injected intraperitoneally (i.p.) at PN5 in the morning.

Galanin Analog administration

NAX 5055 (Robertson et al., 2010; White et al., 2009) was kept at −20°C, light protected until use. 500μg of NAX 5055 were initially diluted in 10μl of dimethylsulfoxide, and subsequently was diluted at the working concentrations with the addition of 1% Tween 20 in normal saline, then left for 30min, light-protected at room temperature, prior to injections. Group assignment to five dose groups was done in a randomized fashion, by an investigator not involved in the animal handling, seizure scoring or outcome assessment. NAX 5055 and its vehicle were administered i.p. as single injections in the afternoon of PN4, one hour after starting the afternoon video monitoring session. We chose to administer NAX 5055 on PN4, so that it is given after the onset of spasms, similar to clinical practice. Each litter was randomized to include pups treated with vehicle as well as pups given each of the 3 different doses of NAX 5055. We used the following abbreviations for the groups of multiple-hit

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pups given NAX 5055 or its vehicle: NAX-0.5: 0.5 mg/kg; NAX-1: 1mg/kg, NAX-2: 2 mg/kg, NAX-4: 4 mg/kg and VEH: vehicle-injected group. Code names were used for the different treatment groups and the investigator injecting the drug or vehicle, scoring the spasms, and assessing outcomes was blinded to the treatment group. In addition, a dose of 7mg/kg NAX 5055 i.p. (NAX-7 dose group, n=6 rats) was tested for tolerability only in PN4 male pups treated according to the multiple-hit model.

Inclusion/exclusion criteria

Exclusion criteria were set *a priori* to exclude rats that were either (a) neglected by the dam, (b) had lesions extending to bilateral hemispheres, (c) did not express spasms prior to the time of drug or vehicle injection, or (d) died as a result of accident (i.e. mechanical injury from the injection or surgery-related trauma or death). Exclusions were made by an investigator blinded to group assignment, prior to the unblinding stage of the study.

Monitoring

At PN4, pups were separated for video monitoring as described in our previous studies (Ono et al., 2011; Raffo et al., 2011; Scantlebury et al., 2010). The single-injection monitoring session at PN4 consisted of one pre-injection and 5 post-injection hours, i.e., six hours total. At PN5, two 2-hour sessions were performed (morning and afternoon). Assessment of pre-injection spasm frequency was conducted during the one hour pre-injection monitoring. “Behavioral spasms” were considered the sudden, synchronous and high amplitude movements of all four limbs and body presenting as flexion, or extension or mixed flexion/extension events. Events that were associated with flexion or extension in an attempt to change position, or with sudden but asynchronous limb movements were not scored.

Weights and neurodevelopmental reflexes were recorded each morning at PN3 to PN5. At PN4 neurodevelopmental reflexes were repeated 2 hours after the galanin analog injection to test for sedation. The battery of reflexes included: (a) open field activity (OFA), i.e. time to escape from a 12.5 cm diameter circular field; (b) negative geotaxis (NG), i.e. time to turn 90° after placed head downwards on a 45° inclined surface, and start climbing up; (c) surface righting time (SRT), i.e. time to turn from the supine position to the prone with the pup standing on four limbs. Maximal observation period for each of these tests was set at 60sec and hence a score of 60sec indicated failure to perform this test within the allotted period of time.

Respiration, heart rates and blood oxygenation by pulse oximetry were assessed using the Mouse Ox system (STARR Life Sciences Corp, USA), whereas blood glucose level was measured using tail blood glucose measurements (Optium kit, Abbott, IL, USA) at 4 hours post-injection.

Nissl staining

The animals were euthanized on PN5, after the end of the afternoon session with pentobarbital (100mg/kg i.p.). The brains were fast frozen in dry ice / 2-isomethylbutane and stored at −80°C. Coronal 40μm sections were stained with thionin staining for histology.

Total RNA purification and qRT-PCR for GalR1, GalR2, and β-actin

To determine the relative expression of GalR1 and GalR2 mRNA in the cortex, we used qRT-PCR. Relative expression was determined over the constitutive gene β-actin. Three male PN4 control rats, 3 PN4 rats treated according to the multiple hit model, and 3 adult male rats (180–200g) were euthanized with 100mg/kg i.p. pentobarbital. Cerebro-cortical samples were collected under sterile conditions at the frontal cortex and retrosplenial

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agranular cortex and through the full depth of the cortex [Figure 166 in (Paxinos et al., 1994)]. These were taken from the right cerebral cortex, spanning 1mm radius from the needle track as well as from the contralateral homotypic cortex. In addition, the anterior dorsal hippocampus was dissected from each hemisphere at the same level as the cortical samples. Samples were collected in RLT buffer (Qiagen Inc, Valencia, CA, USA) with β-mercaptoethanol (10μl/ml), underwent rotor-stator homogenization and total RNA was purified according to the RNeasy Mini kit suggested protocol for total RNA purification from animal tissues with DNAse digestion to remove genomic DNA (Qiagen Inc, Valencia, CA, USA). RNA quantitation was done with a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). All samples had A260/A280 absorbance between 1.93 and 2.06. Reverse transcription polymerase chain reaction (RT-PCR) was done in a Hybaid PCR Sprint machine (Thermo Scientific, Wilmington, DE, USA). The RT-PCR reaction was done using either 100ng of total RNA (for GalR2 qPCR) or 60–300ng of total RNA (for GalR1 qPCR), because of the significantly lower expression of GalR1 versus GalR2 found by our preliminary experiments. We used the TaqMan RT reagents (Life Technologies, Grand Island, NY, USA). The RT-PCR protocol included incubation at 25°C for 10min, followed by 30 min at 48°C, and inactivation at 95°C for 5 min. The qPCR reaction was done using the TaqMan PCR Core reagents, according the manufacturer's protocol (Life Technologies, Grand Island, NY, USA) in a StepOne Real Time PCR System (Life Technologies, Grand Island, NY, USA). The following gene expression assays were used from Life Technologies, which had amplification efficiency of 1 (Grand Island, NY, USA):

- Rat GalR1 the Rn02132426_s1 assay, which amplifies a 115bp within a single exon (exon boundary 1–1, assay location 765). The assay includes a 6-FAM™ dye-labeled TaqMan® MGB probe.
- Rat GalR2 the Rn01773918_m1 assay, which amplifies a 116bp fragment that spans exons (exon boundary 1–2, assay location 386). The assay includes a 6-FAM™ dye-labeled TaqMan® MGB probe.
- Rat β-actin the Rn00667869_m1 assay, which amplifies a 91bp segment spanning exons (exon boundary 4–5, assay location 884). The assay includes a 6-VIC™ dye-labeled TaqMan® MGB probe.

Parallel quantitation of GalR1 and β-actin or GalR2 and β-actin was done, using triplicates of the right and left cerebral cortical or hippocampal samples from each rat. Quantitation was done according to the comparative C_T ($\Delta\Delta C_T$) method as relative expression of GalR1 or GalR2 over β-actin.

Statistics

Based on our prior studies, preliminary power analysis determined that we would need at least 12 rats per group to reach 84% power to detect a difference in the spasms frequencies of 0.22 standard deviations on the log scale between treatments. Data analyses were performed of raw frequencies of spasms, frequencies normalized over the pre-injection frequencies of the same rat, [*normalized frequency = (post-injection frequency/ pre-injection frequency) * 100*] and log-transformed frequencies. The results based on the log-transformed data did not differ materially from those based on the raw or normalized values and therefore are not included. To assess the effects of the drug or vehicle on spasms and neurodevelopmental reflexes, a linear mixed effects modeling approach was used to account for the correlation in repeated measures from the same animal. Fixed effects in the model included time, treatment, and time * treatment interaction terms. Time was modeled as either a categorical or continuous variable, depending on whether the time trends were linear. In the analysis of the non-normalized spasm frequencies, pre-injection levels were included as an additional covariate in the model to adjust for baseline levels. The treatment

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effects on spasms, neurodevelopmental reflexes, weight and vital signs at specific time points were compared with ANOVA (SAS software, SAS Institute, Cary NC, USA). A two-sided p-value less than 0.05 was considered statistically significant.

Results

Exclusions, sample sizes, and histology

We have excluded from the analysis four pups treated according to the multiple-hit protocol. Two animals died post-operatively on PN3; one animal died from hemoperitoneum caused by mechanical injury from the i.p. injection of NAX-0.5 on PN4. One pup (NAX-2 group) was excluded on PN4 because it was neglected by the dam and was found consistently apart from the litter. No rats were excluded based on lesion severity assessed by Nissl staining. Therefore, following these exclusions, from an initial sample size of 11–14 rats/group, we included in this study 13 VEH pups, 12 NAX-0.5 pups, 11 NAX-1 pups, 13 NAX-2 pups, and 11 NAX-4 pups. To determine the separation from a maximally tolerated dose, an additional litter (4 VEH and 6 NAX-7 pups) was included only in the assessment of weights, neurodevelopmental reflexes, cardiorespiratory parameters, and blood glucose.

The typical lesion observed on Nissl staining at the injection site is localized at the peri-infusional region of the right parietal cortex and the adjacent periventricular regions (right anterior-dorsal hippocampus and corpus callosum) (Supplemental Figure 1). This pattern of lesion was found in all pups included in this study with the following exceptions. Three pups (one each from the following groups: NAX-0.5, NAX-2, NAX-4) showed predominant right parietal lesion with no visible injury of the right hippocampus. One pup (NAX-0.5 group) showed predominant right hippocampal lesion with small right parietal lesion. These four pups manifested spasms within the range of frequencies seen in the other rats.

Effects on spasms

Overall, there was no evidence of a treatment effect on the raw or normalized frequencies of spasms when data from all time points were combined in a mixed effects analysis. Time was analyzed as a categorical variable in this analysis since the time trends were not linear over the entire post-injection period. Neither the treatment nor the treatment * time interaction were significant (Table 1). However, pre-injection spasms frequencies were significantly predictive of raw frequencies at post-injection time points, and the time effect was significant for raw and normalized frequencies (Table 1).

When raw frequencies of spasms were compared between treatment groups at specific time points, a significant difference was observed only at 4 hours post-injection (P=0.009, ANOVA) (Figure 1). Pairwise comparisons of the mean raw frequencies of spasms among the different doses showed less frequent spasms in the NAX-4 group compared to each of the other NAX 5055 doses, but not compared to the VEH. Similar results were obtained when the 4-hour values were adjusted for the pre-injection values using analysis of covariance. The rates of change (i.e. slope) in spasm frequencies over the first 4 post-injection hours were also compared between groups (Table 2). The slope in the NAX-4 group was significantly lower compared to the other NAX 5055 treated groups but not compared to the VEH group. In addition, the slope in the VEH group was significantly lower compared with the NAX-0.5 group.

When normalized frequencies of spasms were compared, no significant differences were seen among groups at any time point (Table 2). Furthermore, no significant treatment-related differences in the slopes of normalized values up to the first 4 hours post-injection were observed. In combination, these suggest that NAX 5055 does not acutely suppress spasms compared to vehicle.

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Effects on weights, neurodevelopmental reflexes, glucose homeostasis and vital signs

Treatment had no significant effect on neurodevelopmental reflexes (OFA, SRT, NG) obtained 2 hours post-injection (Table 1, Figure 2). Treatment also had no effect on heart rate, respiratory rate, pulse oximetry, and blood glucose obtained 4 hours post-injection (Table 3) or on daily weights (Table 1). NAX-7 pups showed reduced pulse and breath distention (Table 3) but no other alterations in the other cardiorespiratory parameters, neurodevelopmental reflexes, weights, blood glucose or survival.

Expression of GalR1 and GalR2 in the cerebral cortex and hippocampus

To determine whether the lack of effect of NAX-5055 could be due to absence of GalR1, we quantified the relative expression of GalR1 mRNA in the sensorimotor cortex and hippocampus of PN4 (control and multiple-hit model) and adult male control rats, using qRT-PCR and β-actin as a constitutively expressed control gene. For comparison, GalR2 qRT-PCR was performed in cortical samples of PN4 male rats.

GalR1 mRNA was present in all age and treatment groups, at both regions (Figure 3). A developmental increase in GalR1 mRNA was observed in the hippocampus (more GalR1 in the adults) but not in the cortex. However, GalR1 mRNA was increased in the right hippocampus of the pups treated with the multiple-hit protocol compared to the right hippocampus of the controls.

In PN4 pups, GalR1 mRNA expression in the cortex ranged between 1.5×10^{-6} to 6.7×10^{-6} times the expression of β-actin mRNA (Figure 3). In contrast, GalR2 mRNA ranged between 22.5×10^{-6} to 45×10^{-6} times the β-actin mRNA in the cortex, across groups (Figure 3) [GalR1 versus GalR2; $F_{(7,23)} = 40.45$ (ANOVA, $P<0.0001$)]. Neither the treatment (control versus multiple-hit; $F_{(7,23)} = 0.6$) nor the lateralization (right versus left cortex; $F_{(7,23)} = 1.49$) had any effects on GalR2.

Discussion

We show that a single injection of the GalR1-preferring galanin analog NAX 5055 (0.5–4 mg/kg i.p.), given after the onset of spasms, has no significant acute effect on the frequency of spasms in the multiple hit rat model of IS. GalR1 mRNA was expressed, although at significantly lower doses than either GalR2 in PN4 rats or GalR1 in adult rats. The tolerability of the tested NAX 5055 doses was however good, neurodevelopmental reflexes were not affected, and no other side effects were observed. A significant reduction in pulse distention and breath distention was observed in the NAX-7 group, but without altering the other cardiorespiratory parameters.

We use a single-injection administration paradigm, because the aim of our screening strategy is to find treatments with rapid-onset control of spasms, based on the clinical observations that early cessation of spasms may improve outcomes. We also initiate treatment after the onset of spasms is documented to maintain a clinically-relevant treatment protocol. Currently, the appearance and onset of IS cannot be predicted in infants with IS of non-genetic etiology, which precludes the utilization of a pre-treatment approach.

GalR1 mRNA was present in both the cerebral cortex and anterior dorsal hippocampus in all studied age and treatment groups (Figure 3). GalR1 mRNA expression in PN4 pups was 5.7 times lower than the hippocampal expression of GalR1 in adults, an age when NAX 5055 was able to reduce seizures in adult rodents (White et al., 2009). This finding might imply that the lower GalR1 expression in the peri-infusional cortical and hippocampal regions of PN4 rats could explain the lack of NAX 5055 effect in our model. However, extrapolations across species, strains, ages, as well as among different seizure models need to be done

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cautiously. Further studies in other animal models of seizures in PN4 rats would be useful in testing whether the lack of NAX 5055 in our model is due to the low GalR1 expression in PN4 rats.

The lack of acute efficacy of NAX 5055 in this model of IS, as opposed to other seizure models (White et al., 2009), may also reflect the distinct pharmacosensitivity of spasms from other seizures. This is also reminiscent of our previous results showing no response of spasms to phenytoin (Ono et al., 2011), which is not among the treatments recommended for human IS (Mackay et al., 2004; Pellock et al., 2010). Another possibility is that the lack of observed acute effect of NAX 5055 over spasms may be attributed to the pharmacoresistant nature of spasms in the multiple-hit model (Scantlebury et al., 2010). Spasms in this model do not respond to ACTH and only transiently respond to vigabatrin, rendering the multiple-hit a model of drug-resistant IS (Scantlebury et al., 2010). On the other hand, the good therapeutic effect of the mTOR inhibitor rapamycin (Raffo et al., 2011) and carisbamate (Ono et al., 2011) in the multiple-hit model underlines the utility of this model as a screening tool for the identification of new candidate treatments for IS.

However, our findings cannot exclude the possibility that repetitive administration of NAX 5055 may be needed to suppress spasms in the multiple-hit model, as previously shown for low doses of rapamycin (Raffo et al., 2011). In the adult rodent brain, GalR1 inhibits presynaptic glutamate release by opening G-protein-mediated inward rectifier K+ channels (GIRK) or ATP sensitive K+ channels (Mazarati et al., 2006; Mazarati, 2004; Mazarati et al., 2000). In addition, GalR1 has been shown to inhibit the phosphorylation of *cAMP response element binding protein* (CREB), which can modulate neuronal and synaptic plasticity (Badie-Mahdavi et al., 2005). Further studies utilizing repeat administration of NAX 5055 may be useful to test the effect of prolonged treatment with NAX 5055 on spasms. Even though GalR1 is expressed in low levels in the PN4 cortex, it is possible that repetitive NAX 5055 administration could influence subcortical structures, in which GalR1 expression and signaling may be more potent (Burazin et al., 2000).

Our qRT-PCR results agree with in situ hybridization studies that showed lower GalR1 mRNA expression in the cortex and anterior dorsal hippocampus of PN4-70 rats than in other brain regions such as the ventral hippocampus, limbic regions, thalamus or brainstem (Burazin et al., 2000). Similar to our results, developmental increase in GalR1 mRNA between PN4 and adult rats has also been described in the dorsal dentate gyrus and ventral CA fields of Ammon's horn, but not in the frontoparietal cortex (Burazin et al., 2000). Although GalR1 mRNA expression may not necessarily translate into functional protein, determination of GalR1 protein expression is hampered by the known lack of specificity of the available GalR1 antibodies when tested in knockout mice (Lu and Bartfai, 2009). Using a different anti-GalR1 antibody, we found staining in the cerebral cortex of control and multiple-hit PN4-5 male rats. However, nonspecific staining was also observed in GalR1 knockout mice, which prevented the utilization of these immunochemistry experiments in our manuscript. We report these findings as supplemental material, as a reference on the properties of the specific antibody we used (Supplemental Figure 2). Because of the lack of specificity of the available anti-GalR1 antibodies, we cannot therefore conclude whether differences in GalR mRNA expression correspond to protein expression differences.

Our study also demonstrates that GalR2 mRNA expression was at least 20 times more abundant than GalR1 mRNA in the cerebral cortex of PN4 pups, which is in agreement with a prior study (Burazin et al., 2000). It would therefore be very interesting, in future studies, to determine the effects of GalR2-preferring analogs in the treatment of IS.

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We did not observe any clinically important adverse effects of NAX 5055 doses in PN4 rats, even though the tested NAX 5055 doses exceeded the median toxic dose for adult rats (Bialer et al., 2010). The reduction in pulse and breath distention seen in the NAX-7 group – usually indicative of reduced cardiac output and/or respiratory effort - is of unclear significance. High doses of galanin may lead to hypotension and tachycardia, decreased cardiac output or respiratory suppression through central mechanisms (Abbott et al., 2009; Abbott and Pilowsky, 2009; Diaz-Cabiale et al., 2005) or may have peripheral negative inotropic effects, as shown in guinea pig heart papillary muscle (Kocic, 1998). However, the lack of concomitant alterations in heart or respiratory rates or oxygenation in our study suggests that the observed reduction in pulse and breath distention is not clinically significant. However, a limitation of our experiments is that we only studied one timepoint, so that we do not disrupt our primary endpoint, the monitoring of the spasms. Future experiments should address the time course of the effects of very high doses of NAX 5055 on the cardiorespiratory system, in conjunction with the pharmacokinetics of NAX 5055. Even though our study did not permit the definition of a maximally tolerated dose for NAX 5055 in PN4 rats, further doses were not tested, because of the lack of effect on spasms and the low levels of GalR1 at this developmental age.

Galanin is known to have a regulatory effect on glucose metabolism and inhibits pancreatic insulin secretion in dogs and in rodents, in a stimulus-dependent manner (Gregersen et al., 1994; Hermansen, 1988; Lindskog et al., 1995; Ruczynski et al., 2002; Verchere et al., 1992) mediated through GalR1 (Mitsukawa et al 2008). However, we did not observe any definite hyperglycemic effects of NAX 5055 with any of the utilized doses, which could be due to either the young age of our pups or to missing the timepoint of the peak drug effect on the pancreas. Injection of galanin or its analogs can decrease body core temperature (Mitsukawa et al., 2009; Patel and Hutson, 1996). This possible side effect was not assessed in our study because pups need a controlled thermic environment when separated for monitoring, which makes the analysis of body temperature difficult.

To our knowledge, this is the first time that antiseizure properties of NAX 5055 are tested on a seizure model in immature rodents. We observed no effect on spasms, which could be due to (a) the low GalR1 mRNA expression in cortical and hippocampal regions of PN4 rats, or (b) to the drug-refractoriness of the multiple-hit model of IS or (c) the distinct pharmacosensitivity of IS, or (d) different pharmacokinetics of the drug in neonatal versus adult rats. Although we provide evidence for target expression in the brain of PN4 pups, the lack of effect did not allow us to prove target engagement by the drug. Future studies testing NAX 5055 in other models of seizures in PN4 rats would be useful in differentiating whether the lack of efficacy is model or age-related. Our study was not designed to test the effects of NAX 5055 on long-term epilepsy, cognitive and neurodevelopmental outcomes, which will be important to know as the therapeutic indications of this drug become better described (Crawley, 2008; Mazarati et al., 2006)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ACTH	adrenocorticotropic hormone
GalR	galanin receptor
i.p	intraperitoneal
IS	infantile spasms
NG	negative geotaxis
OFA	open field activity
PN	postnatal day
SRT	surface righting time

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Highlights

- NAX 5055 has no acute effect on spasms in the multiple-hit model.
- NAX 5055 is well tolerated by immature rats.
- The cerebral cortex of postnatal day 4 rats expresses more GalR2 than GalR1.
- Hippocampal GalR1 mRNA expression increases between PN4 to adulthood.

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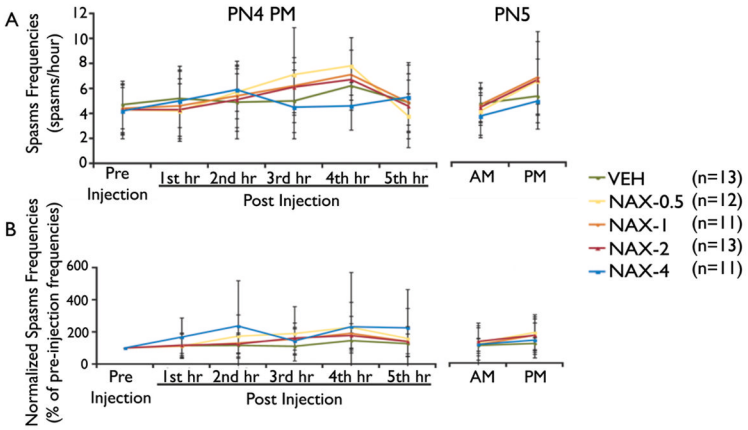

C

	P values [derived from ANOVA comparisons among the different treatment groups at each time point]							
Frequencies of spasms	**1st hr pre injection**	**1st hr post injection**	**2nd hr post injection**	**3rd hr post injection**	**4th hr post injection**	**5th hr post injection**	**PN 5 AM**	**PN 5 PM**
Raw	0.967	0.843	0.847	0.179	***0.009*** *	0.858	0.562	0.294
Normalized	-	0.378	0.248	0.424	0.708	0.648	0.955	0.456

(*) P<0.05

Figure 1. A single dose of NAX 5055 given after the onset of spasms does not suppress spasms in the multiple-hit model (PN4 and PN5)

Panel A. Hourly raw frequencies of spasms after a single injection of NAX 5055 or its vehicle at PN4 in pups treated according to the multiple-hit protocol. NAX-0.5 group had more frequent spasms compared with the VEH group at the 4th post-injection hour. NAX-4 group had less spasms compared to the other NAX groups but not compared to the VEH, at the 4th hour post-injection.

Panel B. Normalized frequencies of spasms (% of pre-injection frequencies of the same rat) after a single injection of NAX 5055 or its vehicle in pups treated according to the multiple-hit protocol. No significant differences were found among treatment groups.

Please see also Tables 1 and 2 for the statistics. Bars represent standard deviation. NAX 5055 dose groups are as follows: NAX-0.5 = 0.5 mg/kg, NAX-1 = 1mg/kg, NAX-2 = 2mg/kg, NAX-4 = 4 mg/kg, VEH = vehicle.

Panel C. The table depicts P values derived from ANOVA comparisons of raw and normalized frequencies of spasms among the different treatment groups at each time point. Only the P-value of the raw frequencies during the 4th post-injection hour is statistically significant. Pairwise comparisons of the mean raw frequencies of spasms among the different doses showed less frequent spasms in the NAX-4 group compared to each of the other NAX 5055 doses, but not compared to the VEH.

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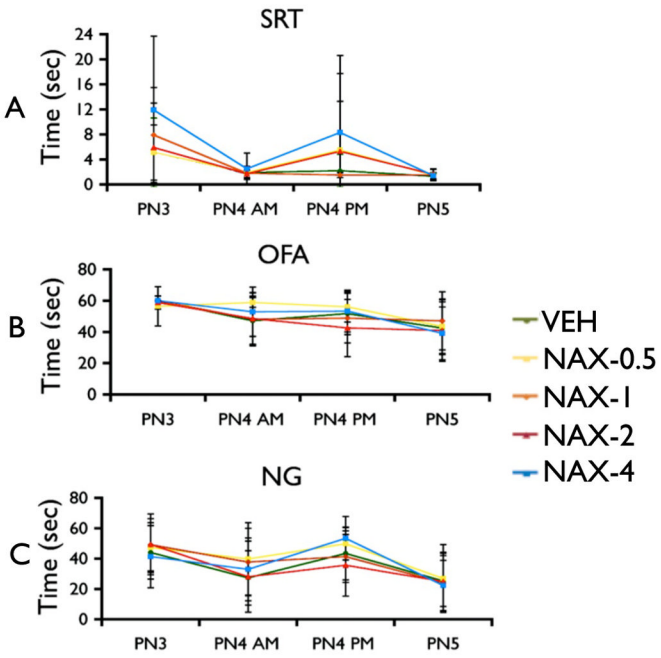

D

	P values [derived from ANOVA comparisons of means among the different treatment groups at each timepoint]			
	PN3	**PN4 AM**	**PN4 PM**	**PN5**
SRT	0.178	0.578	0.341	0.818
OFA	0.510	0.351	0.080	0.886
NG	0.801	0.527	0.281	0.987

Figure 2. NAX 5055 has no significant effect on neurodevelopmental reflexes

Panels A–C. SRT (panel A), OFA (panel B), and NG (panel C) were tested daily in the morning (PN3, PN4AM, PN5) as well as at 2 hours following the NAX 5055 injection (PN4PM). Bars represent standard deviation.

Panel D. The table presents the P values derived from ANOVA comparisons of mean scores for SRT, OFA, and NG among the different treatment groups at each timepoint. No significant differences among groups were seen in these scores.

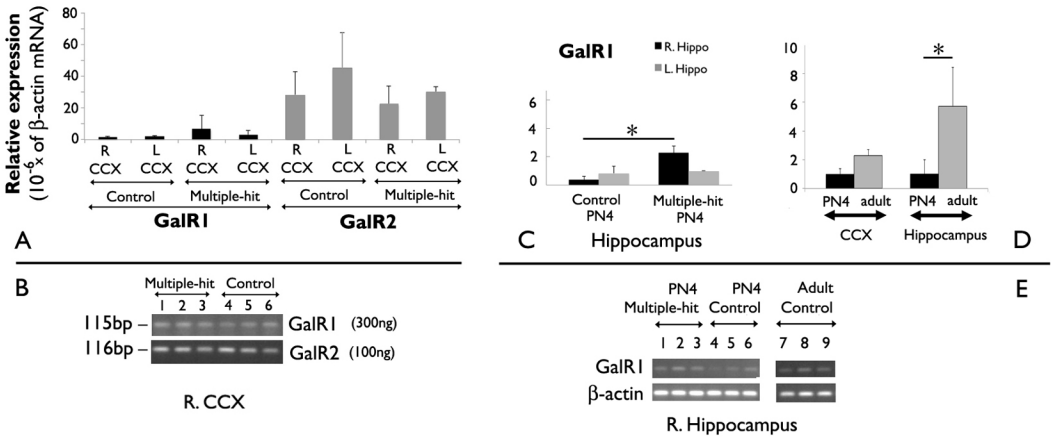

Figure 3. Expression of GalR1 and GalR2 mRNA in PN4 rat cerebral cortex

Panel A: Quantitative RT-PCR of total RNA extracts from cerebral cortex of male PN4 pups (controls or pups treated according to the multiple-hit protocol) revealed significantly more GalR2 mRNA compared to GalR1 mRNA (Gene effect: $F_{(7, 23)}$= 40.45, P<0.0001; n=3 rats per group). There were no inter-hemispheric or treatment-related differences in GalR1 or GalR2 mRNA expression. Results are expressed as 10^{-6} * (β-actin mRNA).

Panel B: The DNA products of the qRT-PCR reactions for GalR1 (115 bp) and GalR2 (116 bp) from right cerebral cortical RNA samples were separated on a Tris-borate-EDTA/ ethidium bromide gel. RT-PCR was performed using 300ng of total RNA for the GalR1 assay and 100ng total RNA for the GalR2 assay.

Panel C: GalR1 mRNA was detected using qRT-PCR in the anterior dorsal hippocampus of both PN4 control rats and rats treated according to the multiple-hit model, but at low levels (n= 3 rats per group). The right hippocampi of the multiple-hit PN4 rats have higher GalR1 mRNA expression compared with the right hippocampi of the controls (Kruskall-Wallis, P<0.05). Black bars indicate right and grey bars the left hippocampal samples.

Panel D: Expression of GalR1 mRNA expression in the cortex and anterior dorsal hippocampus of PN4 and adult control rats, using qRT-PCR. No significant developmental differences are found in the sensorimotor cortex. In contrast, GalR1 mRNA expression increased in the hippocampus of adult control rats compared with PN4 rats (Kruskall-Wallis test, P<0.05; n=3 rats/group; 2 hippocampi per rat).

Panel E: Gel electrophoresis of the qRT-PCR products for GalR1 (115bp) and β-actin (91 bp) from the right hippocampus of nine PN4 and adult rats.

Bars indicate the standard deviations of the mean. The asterisks indicate statistical significance P<0.05. CCX: cerebral cortex; R: right; L: left; Hippo: hippocampus.

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Table 1

Linear mixed model analysis of frequencies of spasms, weights and neurodevelopmental reflexes

	P-values			
	Treatment	**Time**	**Baseline frequencies of spasms**	**Treatment * Time interaction**
Raw frequencies of spasms	0.44	***<0.001***	***0.02***	0.25
Normalized frequencies of spasms	0.48	***<0.001***		0.74
Weights	0.77	***<0.001***		0.67
OFA	0.52	***<0.001***		0.58
SRT	0.07	***<0.001***		0.43
NG	0.69	***<0.001***		0.50

Only pups monitored for spasms are included here.

OFA: open field activity; SRT: Surface righting time; NG: Negative geotaxis.

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Table 2

P-values for pairwise treatment comparisons of rate of change in raw and normalized frequencies of spasms over the first 4 post-injection hours[a].

Treatment Group	Slope Estimate	P (vs. NAX-0.5)	P (vs. NAX-1)	P (vs. NAX-2)	P (vs. NAX-4)
Raw frequencies of spasms					
VEH	0.32	***0.015***	0.205	0.187	0.139
NAX-0.5	1.24		0.268	0.247	< ***0.001***
NAX-1	0.81			0.997	***0.009***
NAX-2	0.80				***0.007***
NAX-4	−0.24				
Normalized frequencies of spasms					
VEH	8.80	0.087	0.300	0.419	0.938
NAX-0.5	36.8		0.531	0.356	0.118
NAX-1	26.13			0.792	0.356
NAX-2	21.72				0.486
NAX-4	10.1				

[a] Adjusted for baseline levels (frequencies of spasms during the 1hr Pre-injection)

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Table 3

ANOVA analysis of vitals and blood glucose levels (PN4PM, 4 hours post-injection)

Test	**P**	**Mean (SD)**					
		VEH	**NAX-0.5**	**NAX-1**	**NAX-2**	**NAX-4**	**NAX-7**
Heart Rate (beats/min)	0.188	399 (55.5)	385.41 (33.89)	358.64 (51.99)	393.09 (52.17)	350.3 (19.12)	383.7 (34.7)
Pulse Distention (μm)	**0.0155**(*)	208.9 (68.2)	239.4 (65.9)	183.7 (41.7)	213.3 (74.3)	231.3 (89.7)	***120.7 (40)***
Pulse Oximetry (%)	0.6432	98.7 (0.59)	97.79 (2.41)	97.79 (3.06)	98.03 (1.45)	86.84 (3.47)	97.5 (2.6)
Respiratory rate (breaths/min)	0.9964	73.6 (19.1)	72.3 (9.37)	72.87 (17.26)	75.3 (9.6)	74.8 (7.6)	74.8 (11.8)
Breath Distention (μm)	**0.043**(**)	313.1 (157.6)	376.6 (119.6)	370.3 (125.5)	319.9 (177.4)	371 (124.6)	***151 (58.6)***
Blood glucose (mg/dl)	0.646	89.6 (20.4)	99 (36.35)	94 (35.28)	107 (37.21)	110 (33.18)	102.8 (5.1)

(*)Significant differences are observed between the NAX-7 group and each of: NAX-05, NAX-2, NAX-4, VEH (Student's t-tests, pairwise comparisons).

(**)Significant differences are observed between the NAX-7 group and each of: NAX-05, NAX-1, NAX-2, NAX-4, VEH (Student's t-tests, pairwise comparisons).