Limits and applicability of behavioral pharmacological models based on the ultrasonic vocalization of rats, and possibilities of further model development

Theses of the doctoral essay

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Introduction

Rats emit ultrasonic vocalization (USV) at three distinct frequency ranges. Adult rats have two vocalization types emitted at 50 kHz and 22-24 kHz, while neonatal rats vocalize at 35-40 kHz. USV plays a pivotal role in communication among individuals.

The 50 kHz USVs can be elicited by positive stimuli and they serve as a vocal signal to find conspecifics. The 22-24 kHz USVs are emitted after negative stimuli, and in certain situations they function as alarm calls (e.g. in presence of a predator). Neonatal rats warn their mother with 35-40 kHz USVs when they separated from the nest.

Anxiety models based on the inhibition of both 22-24 and 23-40 kHz calls are described in the literature. Several compounds were shown to have anxiolytic properties in these models. The playback of natural 22-24 kHz USV or generated ultrasound at the same frequency induce freezing in rats, which behavior is regarded as a measure of fear. A validated anxiety model based on the inhibition of ultrasound induced freezing is not described in literature so far.

Basic aim of behavioral pharmacological animal models is predicting the clinical efficacy of the tested compounds. Knowledge and improvement of the translational value of models is essential for successful drug development. Nowadays, this topic is a neuralgic point of drug research.

In the recent work, the applicability of models based on the ultrasonic vocalization of rats is assessed from certain aspects, and some possibilities for further model development are also presented.

Aims

1. Establishing, comparing and applying methods based on rat USV that appropriate for screen test in the development of anxiolytic drugs

In the applied models, 22-24 kHz USV of adult rats is most often elicited by electric shocking. Three distinct test protocols were established and compared, one without pre-shocking, a multiple shocking and a contextual conditioned USV protocol.

1.1. Finding the appropriate shocking intensity and studying strain differences

Selection of the optimal shocking strength is crucial from both animal welfare and experimental point of view. On one hand, the discomfort of the animals has to be minimized in the experiment, on the other hand the chosen shocking intensity should elicit stable level of USV in the control group. Therefore, the effect of number and current intensity of delivered shocks on the level of USV was assessed. As choosing the appropriate strain for testing is also

an important point, the USV levels of different strains elicited by the same shocking regime was compared. Data on such comparison of rat strains were not found in literature.

1.2. Comparison of test protocols

Based on literature data, the most often applied test protocol is the multiple shocking design. In this paradigm, preceding drug testing animals are exposed to pre-shocking. This design has the advantage that during the pre-shocking sessions non vocalizing animals can be sorted out and pre-shocking increases the level of USV elicited on the test day. The cue and context conditioned paradigms also include pre-shocking, however, these methods are rarely used. Single shocking protocols where pre-shocking is not applied are hardly ever used.

We compared three distinct test protocols, one with multiple shocking design, a context conditioned USV paradigm, and a single shocking protocol in which pre-shocking was not applied. The comparison focused on the effects of selective serotonin transporter inhibitors (SSRI) applied as anxiolytics in the clinic. In the clinical practice, these compounds show anxiolytic and antidepressant effects only after chronic treatment, after acute administration SSRIs are ineffective or anxiogenic. SSRIs were almost exclusively tested in multiple shocking USV protocols. In these paradigms, in contrast to the clinical experiences, SSRIs displayed anxiolytic properties after acute administration. In some of the animal models, anxiogenic effects of SSRIs were detected, while in others, similarly to multiple shocking USV, these compounds had anxiolytic effect. This suggested that SSRIs may display different effects in different USV test protocols.

1.3. Selection of the most appropriate test protocol for screening and its application as a screening test

We aimed to select the most appropriate protocol for screening and test compounds and mechanism of actions which had not studied before in the model.

2. Studying the dual action of CB_1 and $mGlu_5$ antagonism in USV models

A clinical "proof of concept" exists on the antiobesity effect of CB_1 receptor antagonists. However, these compounds cannot be applied in human medical practice due to their severe anxiogenic and depression inducing side effects. However, it is possible, that the anxiogenic side effect of CB_1 antagonists could be eliminated with application of a distinct anxiolytic mechanism. For this purpose, $mGlu_5$ receptors seemed to be potentially appropriate, as $mGlu_5$ receptor antagonists demonstrated wide range anxiolytic actions; furthermore inhibition of these receptors also results in decreased food intake. The major drawback of these compounds is their memory impairing effect. In contrast, memory enhancing effect of CB_1 receptor antagonists was observed in distinct animal models. As some common signalizing pathways

of the two receptors are described in literature, it is possible, that the combination of the two receptor effect results in enhanced food intake inhibiting action which is free from the anxiogenic and memory impairing side effects. We tested this hypothesis with the combined administration of the CB_1 receptor antagonist rimonabant and the $mGlu_5$ receptor antagonist MTEP. For assessing anxiogenic side effect of the combination, a modified version of the single shocking protocol was established which was based on the induction of USV, while memory impairment was tested in the acquisition paradigm of context conditioned USV model.

2.1. Establishing the anxiogenity model based on the induction of USV in adult rats

In USV tests, anxiogenic effects were detected mainly via the elevation of 35-40 kHz USV levels of infant rats, however, these data are rather limited. Even less information is available in literature on increasing of 22-24 kHz USV in adult rats. We made an attempt to establish a model, where low level of USV elicited by low intensity shocking can be elevated by anxiogenic compounds.

2.2. Testing the effect of memory impairing and enhancing compounds on the acquisition of contextual conditioned USV

In contrast to the classical fear conditioning test, conditioned USV paradigms are seldom used for testing fear memory. It cannot be excluded, that these methods are also applicable as memory models. For this purpose, we validated the acquisition paradigm of the contextual conditioned USV model (i.e. when compounds were administered before the shocking trials), by testing the effects of drugs with known memory impairing or enhancing properties.

2.3. Co-administration experiments with MTEP and rimonabant

Effect of the combination on anxiety related behavior was tested in the single shocking and the anxiogenity paradigm, while its effect on memory was tested in the acquisition paradigm of the contextual conditioned USV protocol.

3. Study of freezing behavior induced by 22-24 kHz ultrasound playback

Validated model based on freezing elicited by 22-24- kHz-s ultrasound playback is not described in the literature. We examined, whether the level of freezing induced this way is high enough to allow detecting anxiolytic effects via its reduction. Therefore, freezing induction capabilities of natural USV calls and artificially generated ultrasound playbacks were compared. Furthermore, it was tested, whether voices played back continuously or in bouts induce longer duration of freezing.

Materials and methods

Foot shocks were applied to elicit USV. Shocking was carried out in four 30×30×20 cm plastic wall shocking chambers. Measurement of USV calls was performed with an Ultravox™ system (Noldus Information Technology, The Netherlands).

In the single shocking protocol, animals were placed into the shocking chambers and exposed to foot shocks. USV was measured for 10 minutes right after the last shock. In this paradigm, an animal was used for drug testing only once. We used high shock intensities for testing anxiolytic effects and low shock intensities to assess anxiogenic effects.

The multiple shocking protocol was similar to the paradigm described by Sanchez (Sanchez, 1993), with some modifications. Shocking of the animals was carried out weekly on two consecutive days. Shocking procedure on the shocking days was the same as in case of the single shocking protocol. On the first week, animals were habituated to the experimental protocol, drug tests were not performed. The testing of the compounds started on the second week. The experiment was self controlled, USV measured on the first shocking day of the week was regarded as control. Drug administrations were performed on the second shocking day of the week. Drug testing continued in the same manner on the following weeks. Animals were used for drug testing up to four times. Accordingly, animals were kept in the experiments for five weeks including the week of habituation.

In the contextual conditioned USV paradigm animals were shocked on two consecutive days (training days), the shocking procedure was the same as described at single shocking. On the third day (expression day), animals were replaced to the shocking chambers, but shocks were not delivered. On the expression day USV was measured for 10 minutes right after the replacement to the box. Anxiolytic effects of the compounds were measured by drug-treatment before the third session, while memory impairing or enhancing actions were tested by administering the compounds before shocking on both training days.

At the study of ultrasound induced freezing, the audio files were played with Microtrack II digital recorder. Audio files were edited with Adobe Audition program to produce sound bouts with required length. Behavior of animals was observed in a 35 ×35 ×50 cm plastic box via a camera system. During the 5 minutes long observation period the time spent with freezing was registered. Freezing was defined as total immobility except respiration movements. In this setup, effects of 23 kHz generated ultrasound played continuously or in 2.5, 5, 10, 25, and 50 second bouts were compared. Afterwards, the frequency dependence of the most efficacious sound pattern was tested. Furthermore, its effect was also compared with that of natural USVs played back continuously or in bouts.

Results

1. Establishing, comparing and applying the single shocking, multiple shocking and the context conditioned USV paradigms

1.1. The appropriate shocking intensity and strain differences

- The relation between the shocking intensity and the level of USV is not linear:

In case of delivering 1 or 6 shocks, the USV level displayed a bell-shaped curve with increasing current intensity; when 12 shocks were delivered, the USV reached a plateau. Based on the results 6 shocks with 0.6 mA current intensity was selected for the three protocols.

- Remarkable differences in USV level can be observed among rat strains:

Vocalization levels of Hannover Wistar and Long Evans rats were similar. In comparison, Lister Hooded rats vocalized more, while SPRD rats displayed lower amount of vocalization. Thus, this latter strain is not ideal for USV experiments. Although Lister Hooded rats displayed an outstandingly strong anxiety phenotype, these animals can be purchased for much higher price; therefore in our further studies Hannover Wistar rats were used. In case of this strain, we could not detect significant differences among the different suppliers. In our experiments animals were purchased from Harlan.

1.2. Comparison of test protocols

- The effect of SSRIs considerably differed in the single, multiple and contextual conditioned USV protocols:

In contrast to the benzodiazepine alprazolam, which compound inhibited USV with similar efficacy in all tested protocols, SSRIs were ineffective in the single shocking protocol, showed partial effect in the multiple shocking paradigm, while fully inhibited USV in the contextual conditioned paradigm.

- The three paradigms can be regarded as three different anxiety models:

The results suggest, that in the three protocols USV reflects distinct anxiety states, which can be reduced to a different extent by the same drug.

1.3. Selection and application of the most appropriate test protocol for screening

- Among the three models, the multiple shocking protocol is the most appropriate for screening:

In the multiple shocking protocol, all tested reference compounds significantly inhibited USV, and the measurement capacity of the method provides appropriate testing speed. Its advantage compared to single shocking or context conditioned USV, that in this test animals

can be used multiple times, which is beneficial from both animal welfare and financial point of view.

- Among the 5-HT_{2C}, 5-HT_{5A} and mGlu5 receptor antagonists that were not tested in the multiple shocking model before, only mGluR5 antagonists showed remarkable anxiolytic effect:

The mGluR5 antagonist MTEP and MPEP displayed potent anxiolytic properties, while the 5-HT_{2C} antagonist SB-242084 showed only minimal inhibitory effect. Anxiolytic property of 5-HT_{5A} antagonists was equivocal. Compound A-843277 did not reduce USV, while SB-699551 was efficacious only in a dose-range, where it showed sedative effect in the open field test.

2. Dual action of CB_1 and $mGlu_5$ antagonism in USV models

2.1. Anxiogenity model based on the induction of USV in adult rats

- The novel anxiogenity model is suitable for detecting anxiogenic effect of CB_1 antagonists:

Nor the $GABA_A$ antagonist pentylenetetrazoe, neither the $GABA_B$ antagonist SCH50911 increased USV. In the established protocol, only the CB_1 antagonists, rimonabant and ibipinabant showed significant anxiogenic effect.

2.2. Contextual conditioned USV as a model of memory

- The novel, USV based memory model is suitable for detecting the effect of memory impairing agents:

The NMDA receptor antagonist MK-801 and the $mGluR_5$ antagonist MTEP impaired the acquisition of contextual conditioned USV. In contrast to our expectations, rimonabant did not improve acquisition. It displayed a slight memory impairing tendency instead.

2.3. Effect of co-administrated MTEP and rimonabant

- Due to the expected side effects, development of a dual CB_1-$mGlu_5$ antagonist antiobesity compound is risky:

The combination was not free from side effects. Although MTEP inhibited the anxiogenic effect of rimonabant, the co-administration of the two compounds induced augmented memory impairment.

3. Applicability of freezing behavior induced by 22-24 kHz ultrasound play back as an anxiety model

- Freezing intensity induced by artificial ultrasounds played in bouts is high enough to base an anxiety model on its inhibition:

Artificially generated 23 kHz ultrasounds played in bouts induced higher level of freezing than continuously played sounds. Artificial sounds induced higher level of freezing compared to playback of natural voices. Sounds played in bouts below or over 23 kHz did not significantly induce freezing.

Conclusions

Anxiolytic test based on the foot shock induced USV can be designed with different protocols. In the three studied protocols (single and multiple shocking and context conditioned USV) SSRIs, in contrast to alprazolam, displayed different anxiolytic characteristics. Accordingly, the three test protocols can be regarded as distinct anxiety models with different predictive value.

The three anxiety paradigms cannot be interpreted as disease models. None of them models the main symptoms of a certain anxiety disorder, rather each measures a physiological anxiety response induced by the stress situation generated by the experimental setup. In order to develop disease models, more sophisticated procedures have to be established which accordingly require more time and labor investment. In contrast, the three USV models are appropriate for rapid drug testing, thus they can be applied as screen method in drug development research. Their advantage is that with the combined use of the three models a detailed drug characterization can be performed. Furthermore, the availability of various paradigms offers a possibility of choice to select the most appropriate model for screening a certain group of compounds.

During the establishment of foot shock induced USV models, selecting the appropriate shocking strength is essential. Shocking regime has to be designed on experimental base. During this procedure it has to be taken into account, that the relationship between the strength of the shocking and the elicited USV is not necessarily linear.

Beside the strength of shocking, strain selection is also an important issue, as strain differences in USV levels can be observed under identical shocking regime. Among the tested strains, Wistar rats were found the most appropriate.

Similarly to other anxiety models, the sedative effects of the tested compounds have to be taken into account which can hardly be separated from anxiolysis in the foot shock induced

USV models. The most feasible solution for defining the sedative dose range is testing compounds in models directly measuring sedation.

Beside testing anxiolytic and anxiogenic properties, it is possible to establish USV paradigms where memory impairing effects can also be assessed. The combined use of different USV models provide useful tool for answering complex drug development questions.

Freezing elicited by 22-24 kHz ultrasounds seems to be a promising anxiety model. In contrast to other anxiety models, in this paradigm anxiety state is not elicited by a physically harmful stimulus or by a cue or context predicting that, but by the natural negative extent of 22-24 kHz signals. The validation of this model requires further experiments.

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