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Rats selectively bred for low levels of play-induced 50 kHz vocalizations as a model for Autism Spectrum Disorders: a role for NMDA receptors

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Abstract

Early childhood autism is characterized by deficits in social approach and play behaviors, socio-emotional relatedness, and communication/speech abnormalities, as well as repetitive behaviors. These core neuropsychological features of autism can be modeled in laboratory rats, and the results may be useful for drug discovery and therapeutic development. We review data that show that rats selectively bred for low rates of play-related pro-social ultrasonic vocalizations (USVs) can be used to model social deficit symptoms of autism. Low-line animals engage in less social contact time with conspecifics, show lower rates of play induced pro-social USVs, and show an increased proportion of non-frequency modulated (i.e. monotonous) ultrasonic vocalizations compared to non-selectively bred random-line animals. Gene expression patterns in the low-line animals show significant enrichment in autism-associated genes, and the NMDA receptor family was identified as a significant hub. Treatment of low-line animals with the NMDAR functional glycine site partial agonist, GLYX-13, rescued the deficits in play-induced pro-social 50-kHz USVs and reduced monotonous USVs. Since the NMDA receptor has been implicated in the genesis of autistic symptoms, it is possible that GLYX-13 may be of therapeutic value in the treatment of autism.

Keywords

Autism; Ultrasonic Vocalizations; Selective Breeding; Rats; NMDA Receptor; GLYX-13

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1. Vocal communication in mammals relevant to autism

Vocal communications in mammalian species, especially emotion related forms, have been at the center of research interest for decades because of its complex neural control, biological importance of transfer of information and regulation of behavior, as well as pertinence to the question of their social-emotional processes [1–4] as well as human speech and language evolved [5–7]. Some of the early work on the neurochemical control of separation-calls of mammals and birds, led to the first animal-model based neurochemical theory of autism [8], suggesting aberrant self-attachment to endogenous opioids which led to an opioid-receptor antagonist strategy for treating autism [10]. Indeed the efficacy of low-dose naltrexone was eventually affirmed in several double-blind studies [9–11]. Further development of animal models of autism has been slow, but has recently increased as an understanding of the positive affective aspects of certain mammalian vocalizations has emerged [12].

In recent years, research has focused on rat ultrasonic vocalization (USVs) as a well-developed signalling system of particular importance in communication of emotional states [13–15]. Since rodent vocalizations in the ultrasonic sound frequencies evolved as an adaptive anti-predator strategy [16–17], it allowed social species to develop an elaborate communication system that could not be detected (or if detected, could be poorly localized) by most of the predators [18]. We will focus here on rat ultrasonic vocalization and its role in the expression of emotionality, and share potential implications of such knowledge for development of new treatments of autism. Before proceeding to therapeutic implications, we will first review evidence for the affective aspects of rodent USVs.

1.1 Communicative function of ultrasonic vocalization

Research of the last twenty years has distinguished two basic types of ultrasonic vocalizations in rats associated with emotionally negative and positive states. Aversive vocalizations, termed 22 kHz type USVs, are emitted in dangerous and threatening-to-the-rat situations, or situations causing discomfort, frustration, and significant stress and anxiety. They include proximity of a predator (e.g., a cat) or potential predator (e.g., human), repeated and unpredictable unpleasant or painful stimuli (e.g., air puff, foot shock, application of drugs with aversive properties), withdrawal from prolonged exposure to addictive drugs, losing fight with an aggressive conspecific, unsuccessful mating, and postejaculatory refractory period [16–28].

The second type of vocalization, termed 50 kHz type USVs, includes short calls emitted in a variety of social non-aversive, appetitive, and rewarding situations. They are especially evident during juvenile play or anticipation of play, in response to application or self-administration of addictive drugs, anticipation of rewarding electrical brain stimulation, sexual interactions and expectation of sexual contact, response to familiar conspecifics or anticipation of social contact, particularly after a period of separation, and other appetitive behaviors during mating and aggression [15, 21, 29–42]. Positive affective communicative function of ultrasonic vocalizations was further supported by studies employing playback of ultrasonic vocalizations to rats or by self-administration of vocalizations from a loudspeaker. Playback of 22 kHz vocalizations to naïve rats caused behavioral inhibition, decrease in

locomotor activity, and increase in freezing behavior [43–44]. Additionally, it has been demonstrated that rats attempting to self-administer ultrasonic vocalizations avoided playback of 22 kHz calls [21]. Alternatively, playback of 50 kHz calls caused approach behavior, increased time of exploration in the vicinity of the loudspeaker, and has facilitated sexual encounters [21, 44–46].

These studies have strongly indicated that 22 kHz and 50 kHz vocalizations play communicative role for rats, transmitting biologically significant affective information, and can dramatically influence behaviors of receiver animals. This is particularly well evidenced with emission of 22 kHz alarm calls. In order to inform conspecifics unconditionally about environmental dangers, some higher-order learning experiences are also to be expected. Indeed, association of 22 kHz vocalizations with dangerous situations was shown to require some learning process [47]. Moreover, rats make the association between danger and alarm calls quickly and the aversive memories are resistant to extinction. These results have suggested that rats are genetically predisposed to acquire defensive strategies in response to 22 kHz alarm calls more than to other acoustic stimuli, and animals demonstrate these associations for a longer time [48]. It has been also observed that emission of alarm calls requires an audience effect, i.e., it is dependent on the presence of other conspecifics in the vicinity of the calling rat [49]. Consequently, rats reared individually emitted significantly less 22 kHz alarm calls in response to painful stimuli than rats reared in pairs [50].

1.2 Expression of emotional states by ultrasonic vocalization

Pharmacological studies have provided evidence that instigation of aversive central emotional states concomitant with emission of 22 kHz vocalizations is dependent on activity of the ascending cholinergic system from the laterodorsal tegmental nucleus to the medial mesencephalic and forebrain regions [51–55] while the initiation of an affectively positive appetitive states with concomitant emission of 50 kHz vocalizations is dependent on the activity of the ascending dopaminergic system from the ventral tegmental area to the nucleus accumbens [14, 56–57]. The 22 kHz and 50 kHz calls have distinct and non-overlapping acoustic structures allowing receivers to unambiguously distinguish these calls [14]. Rats emitting 22 kHz or 50 kHz vocalizations 1) communicate their emotional state to conspecifics that are present nearby, 2) display social importance, and 3) have adaptive value for rats [14]. For example, it was reported that rats vocalize more during social contacts than in solitude [32, 58], and that subordinate rats emitted significantly more 22 kHz alarm calls than dominant animals [50].

Emission of calls directed to other rats also means that the receivers of the vocalizations should be able to recognize the emotional state of the signallers by listening to their calls in order to make relevant behavioural decisions. Indeed, studies of c-Fos expression in the brains of the receivers in response to playback of 22 kHz or 50 kHz calls labelled different regions of the receivers' brains [46], while previous work had already shown that the aversive calls selectively aroused established negatively affective circuits such as those in the dorsal periaqueductal gray [59]. C-Fos protein could be detected in the frontal association cortex and nucleus accumbens in response to the playback of 50 kHz calls, the brain regions related to reward. On the other hand, playback of 22 kHz calls caused

increased c-Fos labelling in the perirhinal cortex, amygdalar nuclei, and the periaqueductal gray, areas associated with aversive responses [46].

The association of emitted calls with the emotional state of the signaller suggested that selecting rats on the basis of their calls might also select individuals representing different emotional and motivational phenotypes. Selection of emotional phenotypes can also be achieved by other behavioral features, for example shuttle-box avoidance in Roman high- and low-avoidance rats [60–61].

2. Selective breeding for low and high emission of appetitive 50 kHz vocalizations

Selective breeding of Long-Evans rats for low and high levels of play-induced 50 kHz vocalizations was based on heterospecific play of juvenile rats with the human hand (dubbed “tickling”) [33, 36, 62]. Tickling play, which resembles natural rough-and-tumble play of juveniles, was found to have highly rewarding properties for rats [62] and caused emission of high numbers of 50 kHz vocalizations during the play. Rats were subjected to 2-min tickle play tests consisting of four cycles of 15 s bouts of play followed by 15 s of no stimulation. The tests were performed once a day for four consecutive days. The “tickling” procedure included rapid finger movements on the back and neck of the rat, interspersed with quick turning of animals on their backs with continued tickling. Namely rats were subjected to fast and vigorous finger movements first over their dorsal surfaces and then ventral surfaces after which rats were momentarily released, and the sequence was repeated. This short behavioural assay could be effectively used to index stable affective phenotypes and relevant brain regions, across laboratories [e.g., 63–64] that could serve as a reasonable genetically-informative starting point for an affectively-based medicinal discovery program [65] based on the genetic selection of different social-affective phenotypes as described below.

Rats were engaged in tickling starting at 24 days of age for four days and then selected into three groups for further breeding: high vocalizing (high line), low vocalizing (low line) and random (random line, not selected control group) [36]. The number of 50 kHz vocalization (of any type) emitted on the fourth day was taken for selection. Rats from the high line were bred only within rats from the same line, rats from the low line only with rats from the low line, and random line rats were bred with rats within their line. For the random line, animals were chosen randomly without further selection. Rats were bred by cross breeding, i.e., selected males and females were originating from different litters of the same line [36].

2.1 Differences in emotional phenotypes among the selected lines of rats

After the first four generations, high line rats emitted significantly more appetitive (pro-social) 50 kHz calls than random line rats, while low line rats did not differ from controls. Starting from the 9th generation, low line rats consistently showed significantly lower emission of 50 kHz calls than the random line rats [66].

Rats of the high and low lines also differed in the number of emitted aversive 22 kHz calls. High line rats emitted significantly less 22 kHz calls than random line rats from the second

generation. After the 7th generation, high line rats practically stopped emitting any 22 kHz vocalizations. On the other hand, low line rats emitted significantly more aversive 22 kHz vocalizations than random line rats from the 9th generation [66]. This reciprocal relationship between high and low lines in emission of appetitive versus aversive vocalizations is illustrated on a summary diagram over 25 generations (Fig. 1). Since the 13th generation, emission of vocalizations roughly stabilized. On average, high line rats emitting 4-fold more 50 kHz calls than low line rats, and low line rats emitted 23-fold more aversive 22 kHz calls than high line rats (Fig. 1). Both these levels significantly differed from the random line controls.

Further studies have revealed that the low line rats had greatly changed behavior since infancy. The low line rats showed increased number of juvenile isolation calls and lack of preference for maternally associated odor in the place preference test, while high line rats did not differ consistently from the control [67]. Also, the natural rough-and-tumble play of juvenile rats differed among the lines. High line rats executed more dorsal contacts and low line animals more pinning behavior during their play [68]. Moreover, the high line rats showed increased suppression of juvenile play after exposure to cat odor as compared to low line animals. This observation led to the conclusion that high and low line rats differ in their emotional arousal [68].

Low line rats showed a permanent change in their emotional behavior and, in adulthood, they did not demonstrate curiosity characteristic for the high line animals when they were exploring the center of an open field [66]. Adult high line rats were significantly more active in an activity box than low line rats both in the control conditions and after intraaccumbens application of amphetamine [54]. The low line rats also showed a significantly increased number of fecal boli during an open field test or heterospecific play, as compared to controls, suggesting an increased autonomic activity [66]. While amphetamine-induced increase in locomotor activity compared to baseline activity represented similar proportion across all the lines, the number of intraaccumbens amphetamine-induced 50 kHz vocalizations was dissimilar in the low and high line rats. While rats of the low line did not respond significantly to intraaccumbens amphetamine, rats of the high line showed a robust increase in the number emitted 50 kHz calls that was 3.4 times higher than that in the random line and 6.4 times higher than that in the low line rats [69].

The low line animals have demonstrated significant social deficits. They not only emitted less pro-social 50 kHz calls but they showed less contact time with conspecifics in the social contact compartment of a social interaction apparatus compared to random rats. They also exhibited decreased aggressiveness and inflicted fewer bites to the residents in a social defeat test [66]. These features, and particularly low levels of pro-social 50 kHz calls, increased levels of aversive, anxiety-based 22 kHz calls, low motor and locomotor activity strongly suggest that the low line rats exhibited features of social withdrawal and decreased social contacts, which can be used to model depression [66]. These symptoms are also compatible with those observed in individuals with Autism Spectrum Disorders (ASD) [12].

2.2 Low-line rats show an autism-like phenotype and gene expression patterns

As already noted, the core symptoms of ASD are: (1) deficits in social emotions and motivations [e.g., 70], (2) communication problems, particularly features of vocal communication [71–72], and (3) a high incidence of motor abnormalities, especially repetitive and restrictive behaviors [73]. These symptoms can be modeled in part in animals using the following behavioral measures, respectively: (1) reduced time spent in social contact; (2) reduced rates of pro-social vocalizations, and (3) more indirectly, disrupted ultrasonic vocalization patterns [74].

Low line animals engage in less social contact time with conspecifics, show lower rates of play induced pro-social ultrasonic vocalizations, and show an increased proportion of monotonous ultrasonic vocalizations compared to randomly bred animals when tested as adults [12]. There is statistically significant overlap between the human autism associated genes and genes differentially expressed in low-line animals [12].

3. NMDA receptors play a functional role in autism

Among many transmitter systems, glutamatergic NMDA receptors have been implicated in etiopathology of several neuropsychiatric disorders including neurodevelopmental disorders such as ASD [75]. The NMDA receptor family was also identified as a significant ASD hub [12], i.e., genes having a greater number of protein-protein interactions with other autism candidate genes. Recently, the glutamatergic receptor family, particularly N-methyl-d-aspartate receptors (NMDAR), have become of interest as targets for the development of autism therapy. Based on neuroimaging and neuroanatomical studies together with the observation that NMDAR antagonists produce autistic-like behaviors in healthy subjects, Carlsson has proposed that infantile autism is a hypoglutamatergic disorder [76]. Post-mortem studies have also shown abnormal glutamate receptor mRNA and protein levels in the cerebella of autistic individuals [77]. The NMDA receptor antagonists, PCP and ketamine, given acutely have been shown to mimic the symptoms of autism in humans [76]. A recent study with autistic individuals showed that daily doses of d-cycloserine (DCS), an NMDAR glycine-site partial agonist, significantly improved social withdrawal [78]. Further, two studies have reported that daily doses of the NMDA receptor noncompetitive antagonist, amantadine (memantine) reduced some of the negative symptoms of autism such as hyperactivity [79, 80].

3.1 GLYX-13 reverses the autistic-like symptoms of low-line animals

GLYX-13 is an amidated tetrapeptide (threonine–proline–proline–threonine) derived from one of the hypervariable regions of a monoclonal antibody, B6B21, shown to be a glycine-site partial agonist of the NMDAR [65, 81–88]. GLYX-13 also behaves as an NR2B subunit preferring NMDAR glycine-site partial agonist [87].

To date, GLYX-13 has been reported to: (1) enhance the magnitude of long-term potentiation of synaptic transmission while reducing long-term depression [87]; (2) significantly increase learning in a variety of hippocampus-dependent learning tasks including trace eyeblink conditioning and the Morris water maze in both young adult and learning-impaired aging rats [81]; (3) have anti-nociceptive properties in both the rat

formalin model of pain and the rat constriction nerve injury model of neuropathic pain at doses that do not induce ataxia [86]; (4) markedly reduce delayed (24 h) CA1 pyramidal neuronal cell death produced by bilateral carotid occlusion in Mongolian gerbils when administered up to 5 h post-ischemia [84] ; and produced robust antidepressant-like effects in the rat Porsolt, Learned helplessness, and novelty induced hypophagia tests [88]. Recently, a Phase 1 clinical trial was conducted that showed that GLYX-13 did not produce any ketamine-like dissociative side effects.

There are several ways to define a partial agonist. For our purposes, a partial agonist is a compound that when added in the presence of a low concentration of a full agonist produces further activation of the NMDA receptor/ion channel complex. However, at high concentrations of a full agonist, the addition of a partial agonist will inhibit the NMDA receptor/ion channel complex.

Evidence that GLYX-13 is a partial agonist at the glycine site of the NMDAR comes from a variety of data. Firstly, GLYX-13 was derived from one of the hypervariable regions of the light chain of a monoclonal antibody shown to act as an NMDA receptor glycine site partial agonist. GLYX-13 was identified by measuring ^3H -MK801 binding to rat hippocampal membrane preparations in the presence of saturating concentrations of the NMDAR glycine-site specific competitive antagonist, 7-chloro-kyneurenic acid. GLYX-13 was found to be able to override this block in a concentration-dependent manner and in a similar fashion to d-cycloserine, a well-established NMDAR glycine site partial agonist [83]. Secondly, GLYX-13 was examined for its partial agonist properties by direct current analyses performed using NMDARs expressed in frog oocytes. GLYX-13 activated these currents in the absence of glycine but in the presence of the coagonist glutamate, inhibited currents in the presence of optimal concentrations of glycine, competed with 7-chloro-kyneurenic acid all in a dose dependent fashion [83], exactly as expected for a glycine site specific partial agonist. (GLYX-13 activated these currents in the absence of glycine. Thirdly, GLYX-13 shows similar behavior using rat hippocampal slices and measuring both NMDAR currents and the enhancement of long-term potentiation [87]. Fourthly, behavioral studies have shown that low concentrations of GLYX-13 enhance learning in a variety of hippocampus-dependent learning tasks, clearly an agonist phenomenon [81], and produces an analgesic effect in rat neuropathic pain models, which is an antagonist-dependent phenomenon [86]. Finally, using whole cell patch clamp technology to measure NMDAR currents in hippocampal slices and following the Stephenson protocol for the identification of partial agonists, it was found that GLYX-13 acted as a partial agonist by facilitating currents in the presence of low concentrations of d-serine and inhibiting currents at high concentrations of d-serine [88]. These studies also revealed that GLYX-13 has approximately 25% of the intrinsic efficacy as d-serine as well [88]. Thus GLYX-13 in pharmacological, physiological and behavioral studies showed typical partial agonist properties at the glycine site of the NMDAR.

GLYX-13 is efficacious in reversing the autism-like phenotype of low line rats. We have shown that GLYX-13 (50 mg/kg, SC) significantly increased rates of play-induced pro-social USVs and significantly decreased the proportion of total USVs that are monotonous [12; figure 2–3]. Whereas, frequency modulated 50-kHz calls have been shown to be

associated with pro-social positive affective states [65], monotonous calls have been associated with communication deficits [89–91]. These results show that GLYX-13 can reverse the social/emotional communication deficit in low-line rats. In addition, microarray studies of low line animals showed that NMDAR function was disrupted in these animals, suggesting that low line rats have autism-like features and may be used as a model of ASD modeling social withdrawal and impairment in social communication. Further studies examining the effects of GLYX-13 on other behavioral measures relevant to autism (e.g. social contact time, and other repetitive behaviours), as well as a more fine-grained analysis of different FM 50-kHz USVs call types [92] are warranted.

4. In Conclusion: autistic-like animal phenotypes and drug discovery

Future developments in preclinical modelling of psychiatric disorders will need to take the affective feelings of animals ever more seriously, since the data-base is substantial that all animals homologously share certain fundamental emotional feelings that evolved long before the human line diverged from other mammals [10, 93]. Although it is foolish to suggest that these ancestral feelings are identical across species, the evolutionary continuities at neuroanatomical, neurochemical and basic affective levels may remain so closely related that a close neurochemical and neurogenetic study of the subcortical brain substrates of affective feeling processes of other animals may help illuminate the human condition, and thereby promote the discovery of more specific mind medicines [94–95].

Our earlier work with the idea that autism may have endogenous opioid self-addictive processes (as opposed to attachment to social others), led to the evaluation of low-dose naltrexone as a treatment modality [10]. In combination with social support, the resulting state induced was a more active pro-social stance in many autistic children. Although it was by no means close to a cure, it promoted positive social interactions and thereby real benefits for more positive forms of family life. We anticipate that the GLYX-13, which has been shown to facilitate positive emotional learning in rats [65,94–95] will also have beneficial effects in autistic children, especially when used in socially-sensitive ways.

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Highlights

The NMDA receptor complex plays a functional role in autism.

Rats bred for low rates of hedonic ultrasonic calls show an autistic-like phenotype.

GLYX-13, a NMDAR glycine-site partial agonist, rescues this autistic-like phenotype.

GLYX-13 may have therapeutic potential for the treatment of autism.

Fig. 1. Selective breeding for high and low rates of rough-and-tumble play induced 50-kHz USVs Number of emitted 50 kHz (**A**) and 22 kHz (**B**) vocalizations by rats of selected lines (HIGH LINE, RANDOM LINE, and LOW LINE) over 5–25 generations. Vocalizations were recorded over 2 min tests with tickle play tests on the 27th postnatal day. All high line scores for 50 kHz vocalizations (**A**) are significantly higher than those for random line rats ($p < 0.5$ to 0.001). All low line scores for 22 kHz vocalizations (**B**), except that for 20th generation) are significantly higher than those for random line rats ($p < 0.05$). Results for generation 5–

13 were taken from previous publication [66]. Vertical bars represent SEMs. $N = 18$ for each line and generation, except high line of the 20th generation with $N = 20$.

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Fig. 2. Rats selectively bred for low rates of 50-kHz USVs show an autistic-like phenotype
 Mean $\pm$ SEM (A) time spent in direct social contact in adult male Long Evans rats selectively bred for low or random rates of play-induced pro-social USVs. Animals were tested in pairs with a conspecific of the same selectively bred line. (B) Rates of conspecific play-induced pro-social USVs (frequency-modulated 50 kHz calls) in adult male low- and random-line animals. (C) Proportion of total conspecific play-induced USVs that are monotonous (i.e., bandwidth less than 7 kHz; Burgdorf et al., 2008). Data adapted and reanalyzed from [66,12]. n = 14–20 per group. *P < .05 Fisher PLSD post hoc test, 2 tailed.

Fig. 3. GLYX-13 reverses the autism-like phenotype of low-line rats

Mean $\pm$ SEM (**A**) rates of conspecific play-induced pro-social USVs (frequency-modulated 50 kHz calls) in adolescent male low-line rats (that exhibit an autism-like phenotype) pretreated with vehicle (1 mg/ml sterile saline s.c.) or GLYX-13 (50 mg/kg s.c.) 15 min before the start of testing using a within-subjects design. (**B**) Proportion of total conspecific play-induced USVs that were monotonous (i.e., bandwidth less than 7 kHz) [21]. Data adapted and reanalyzed from [66,12]. $n = 9$ per group. * $P < .05$ Fisher's PLSD post hoc test, 2 tailed.