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Adult siRNA-induced knockdown of $mGlu_7$ receptors reduces anxiety in the mouse

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ABSTRACT

Our knowledge regarding the molecular pathophysiology underlying anxiety disorders remains incomplete. Increasing evidence points to a role of glutamate in anxiety. The group III metabotropic glutamate receptors ($mGlu_4$, $mGlu_6$, $mGlu_7$ and $mGlu_8$ receptors) remain the least investigated glutamate receptor subtypes partially due to a delay in the development of specific pharmacological tools. Early work using knockout animals and pharmacological tools aimed at investigating the role of $mGlu_7$ receptor in the pathophysiology of anxiety disorders has yielded exciting yet not always consistent results. To further investigate the role this receptor plays in anxiety-like behaviour, we knocked down $mGlu_7$ receptor mRNA levels in the adult mouse brain using siRNA delivered via an osmotic minipump. This reduced anxiety-like behaviour in the light–dark box coupled with an attenuation of stress-induced hyperthermia (SIH) and a reduction of the acoustic startle response (ASRs) in the fear-potentiated startle paradigm (FPS). These effects on anxiety-like behaviour were independent of any impairment of locomotor activity and surprisingly, no behavioural changes were observed in the forced swim test (FST), which is in contrast to $mGlu_7$ receptor knockout animals. Furthermore, the previously reported epilepsy-prone phenotype seen in $mGlu_7$ receptor knockout animals was not observed following siRNA-induced knockdown of the receptor. These data suggest targeting $mGlu_7$ receptors with selective antagonist drugs may be an effective and safe strategy for the treatment of anxiety disorders.

1. Introduction

Anxiety disorders represent a large burden for affected individuals and society in general (Andlin-Sobocki et al., 2005; Nutt, 2005). Available treatments mainly target the serotonergic and GABAergic systems, but are of limited efficacy in a significant proportion of patients and not devoid of side effects (Baldwin et al., 2005; Nutt, 2005). Little progress has been made in the development of novel treatments since the introduction of benzodiazepines in the 1960s and serotonergic drugs in the 1980s (Cryan and Sweeney, 2011). This can be partially attributed to the paucity of information regarding the molecular underpinnings of anxiety disorders.

Accumulating evidence suggests a role for the glutamatergic system in stress-related psychiatric disorders, particularly the metabotropic glutamate receptors (mGlu receptors) (Musazzi et al., 2012; O'Connor and Cryan, 2010; O'Connor et al., 2010; Swanson et al., 2005). The group III mGlu receptors ($mGlu_4$, $mGlu_6$, $mGlu_7$ and $mGlu_8$ receptors) particularly $mGlu_7$ receptor, are among the least investigated largely due to a lack of sufficiently selective drugs.

$mGlu_7$ receptor deficient mice were shown to have deficits in amygdala-dependent fear learning (Masugi et al., 1999) and possess an anxiolytic and antidepressant-like phenotype (Callaerts-Vegh et al., 2006; Cryan et al., 2003b; Stachowicz et al., 2008). Moreover, $mGlu_7$ receptor deficient animals have a dysregulated hypothalamic–pituitary–adrenal (HPA) axis and increases in hippocampal BDNF levels, which are also observed following chronic treatment with anxiolytics or antidepressants (Mitsukawa et al., 2006).

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Certain caveats must be considered when interpreting data from constitutive knockout animals (Crawley, 1999; Gerlai, 2002; Thakker et al., 2004), such as compensatory developmental effects, therefore it is imperative to compare the effects of such genetic manipulations with those produced by pharmacological manipulations in adult animals.

The first $mGlu_7$ receptor selective allosteric agonist, AMN082, has been discovered recently and was characterized in many behavioural and physiological tests (Mitsukawa et al., 2006). In the light of earlier studies using knockout mice, results obtained with AMN082 were somewhat paradoxical. AMN082 produced an anxiolytic effect in the 4-plate test and stress-induced hyperthermia (SIH) (Stachowicz et al., 2008) as well as restoring the extinction of fearful memories in a genetic model of impaired extinction (Whittle et al., 2013). Furthermore, an antidepressant effect in the forced swim test (FST) (O'Connor and Cryan, 2013; Palucha-Poniewiera et al., 2010; Palucha-Poniewiera and Pilc, 2013; Palucha et al., 2007), tail suspension test (TST) (Bradley et al., 2012) and in differential reinforcement of low rates of responding (DRL)-30 (Bradley et al., 2012) was observed following AMN082 administration. This results in a conundrum as to how can both pharmacological activation and genetic ablation induce the same effects?

To address this apparent paradox we have developed an osmotic minipump paradigm which allows the delivery of siRNA to the adult brain resulting in selective knockdown of mRNA/protein levels (Thakker et al., 2004, 2005). This technique was used to produce effective knockdown of the dopamine and serotonin transporters, as well as $mGlu_7$ receptor (Fendt et al., 2008; Thakker et al., 2004, 2005); the latter resulted in an impaired extinction of conditioned taste aversion (Fendt et al., 2008) confirming previous knockout data (Masugi et al., 1999). To further elucidate the role of the $mGlu_7$ receptor in anxiety and depression-related behaviours, the current study employed this delivery system allowing the knockdown of $mGlu_7$ receptor in the adult brain in parallel with several behavioural tests to assess innate anxiety, conditioned fear and depression-like behaviour.

2. Materials and methods

2.1. Animals

Male mice were obtained from Charles River (L'Abresle, France) and were tested within 2 weeks of arrival at the laboratory. BALB/c mice were used in all experiments as the siRNA protocol was initially optimised for this strain (Thakker et al., 2004) except for the fear-potentiated startle paradigm and for EEG recordings whereby these paradigms have been optimised for DBA and C57BL/6 mice respectively (Fendt et al., 2009; Risbrough and Geyer, 2005). Animals were housed two per cage prior to surgery and one per cage thereafter in macrolon cages. Mice received one Mouse House (Nalgene) per home cage and tissue paper nesting materials. Housing was at a constant room temperature of 22–24 °C in a 12 h light/dark cycle with lights on at 6 a.m. Food pellets and tap water were available ad libitum. The range of animal weights at testing over all experiments was 21–34 g. All animal experiments were conducted during the light phase. A radio was used to provide acclimatising and background noise prior to and throughout all experimentation. Three different cohorts of mice were used. Cohort one was tested in the SIH, light–dark box, FST and locomotor investigations. Cohort two was tested in the fear potentiated startle assay and cohort three was tested in the epilepsy model. All experiments were conducted according to international guidelines for the care and use of laboratory animals, with respect to national laws on animal use and he European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimise animal suffering, to reduce the number of animals used, and to utilise alternatives to *in vivo* techniques, if available. The local ethics committees (Kantonales Veterinäramt Basel, Basel, Switzerland) approved all experiments.

2.2. siRNA induced knockdown of $mGlu_7$ receptor

We have previously validated an siRNA based technique for the knockdown of specific mRNA (Thakker et al., 2004). Surgeries were carried out as described previously (Fendt et al., 2008; Thakker et al., 2004) by stereotaxically placing the cannula for continual infusions into the dorsal third ventricle from a subcutaneously implanted osmotic mini-pump reservoir. Infusions performed were of vehicle alone, two different siRNAs specifically targeting the $mGlu_7$ receptor mRNA (GenBank accession no. BC080315.1) at nt 1301–1321 (siRNA-1), 2377–2397 (siRNA-2) or control siRNA sequences to account for any potential effects of nucleotide infusion. Four separate control siRNA sequences were used in total with two of these being used in each experiment; siRNA with a scrambled sequence (scrRNA), 5′-CCUAUGAACGUUAUGACGATT-3′ and siRNA-pGl3 (siRNA targeting luciferase) were used for stress-induced hyperthermia (SIH), light–dark box, monitoring of locomotor activity and the forced swim test (FST). Nucleotide-mismatch siRNAs (mmRNAs) with guide sequences 5′-UGAAUUAAGAAUCCAAUCCdTdT-3′ (mmRNA-1) or 5′-UAAACGGGAUGUAAGUGCCdAdG-3′ (mmRNA-2) were used for experiments involving electroencephalography (EEG) recordings, Pentylenetetrazole-induced seizures and fear potentiated startle (FPS). siRNAs used *in vivo* were selected from a standardized *in vitro* mRNA fusion-construct screening of 11 different constructs with other target $mGlu_7$ receptor sequences at nt 1243–1263, 1442–1462, 1605–1625, 1998–2018, 2196–2216, 2260–2280, 2304–2324, 2445–2465 and 2739–2759 (data not shown) (Huesken et al., 2005). Treatments were pseudorandomly allocated, and the experimenter was blind to the treatments received by the animals. Animals were allowed to recover for 12 days prior to any behavioural tests being conducted. This also provided ample time for the siRNA to induce an appropriate knockdown of $mGlu_7$ receptor as has been previously determined (Fendt et al., 2008).

2.3. Stress-induced hyperthermia

12 days after minipump implantation BALB/c mice were used to assess the effect of $mGlu_7$ receptor manipulation on SIH. SIH was conducted as described previously (Cryan et al., 2004). Mice were singly housed in small macrolon cages 24 h before testing began. Rectal temperature of test subjects was recorded twice i.e. $t = 0$ (T_1) and at $t = 15$ (T_2). Recording of T_1 measures the baseline temperature and also acts as the stressor. Measuring T_2 allows the determination of SIH defined as the difference between T_2 and T_1.

2.4. Light–dark box

BALB/c mice were used to assess the effect of $mGlu_7$ receptor manipulation in the light–dark box. The light–dark box test procedure was carried out as described previously (Cryan et al., 2003a; Finger et al., 2010), 13 days after minipump implantation. The apparatus consisted of a rectangular box (44 × 21 × 21 cm) divided into an open white compartment (30 × 22 × 22 cm) and a black closed compartment (14 × 21 × 21 cm). Access between the two compartments was allowed via a small rectangular opening (12 × 5 cm). The sides of the box were made of clear plastic and a 50 W desk lamp illuminated the box from above. The test animal was placed in the light section of the apparatus facing away from the opening between the two different sections. The animal was allowed to freely explore the apparatus for 10 min. Time spent in the light section, transitions between sections (defined as all four paws entering a section) and latency to leave light section were recorded. Freezing, defined as the total absence of movement of the body with the exception of movements required for respiration, was also recorded. Between subjects the apparatus was thoroughly cleaned. All behavioural scoring was conducted by a highly trained and experienced observer with a high intra-rater correlation across previous experiments.

2.5. Measurement of locomotor activity

On the 15th day following minipump implantation, locomotor activity was assessed in BALB/c mice essentially as described previously (Mombereau et al., 2004). Animals were placed in automated locomotor activity cages (31 cm length, 19 cm width, 16 cm height; TSE, Bad Homburg, Germany) and the distance travelled was measured by the number of horizontal beam-breaks as previously described. Data were in 5 min intervals.

2.6. Forced swim test

14 days after surgery BALB/c mice were used to assess siRNA induced $mGlu_7$ receptor knockdown on depression-like behaviour in the FST. Procedure was performed as described in Thakker et al. (2005). Briefly, Mice were individually placed into plexiglas cylinders (24 cm high 21 cm internal diameter) filled with water (25.4 ± 0.7 °C) to a depth of 15 cm. Test sessions were recorded by a video camera positioned directly above the cylinders. A well-trained observer, blind to the treatment groups, scored these videotapes for the duration of mouse immobility during the last 4 min of the 6 min test period. A mouse was judged to be immobile when making only those movements necessary to keep its head above water. All behavioural scoring was conducted by a highly trained and experienced observer with a high intra-rater correlation across previous experiments.

2.7. In-situ hybridisation

BALB/c mice were decapitated within an hour of their final testing session. Brains were removed and serial coronal sections of 10 μM thickness were obtained at the following anterior–posterior (AP) coordinates, in millimetres, relative to Bregma; (Paxinos and Franklin, 2001) 3.56 (granular layer of the olfactory bulb), 2.46