mu-Opioid (MOP) receptor mediated G-protein signaling is impaired in specific brain regions in a rat model of schizophrenia

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ABSTRACT

Schizophrenia is a complex mental health disorder. Clinical reports suggest that many patients with schizophrenia are less sensitive to pain than other individuals. Animal models do not interpret schizophrenia completely, but they can model a number of symptoms of the disease, including decreased pain sensitivities and increased pain thresholds of various modalities. Opioid receptors and endogenous opioid peptides have a substantial role in analgesia. In this biochemical study we investigated changes in the signaling properties of the mu-opioid (MOP) receptor in different brain regions, which are involved in the pain transmission, i.e. thalamus, olfactory bulb, prefrontal cortex and hippocampus. Our goal was to compare the transmembrane signaling mediated by MOP receptors in control rats and in a recently developed rat model of schizophrenia. Regulatory G-protein activation via MOP receptors were measured in [^{35}S]GTPγS binding assays in the presence of a highly selective MOP receptor peptide agonist, DAMGO. It was found that the MOP receptor mediated activation of G-proteins was substantially lower in membranes prepared from the 'schizophrenic' model rats than in control animals. The potency of DAMGO to activate MOP receptor was also decreased in all brain regions studied. Taken together in our rat model of schizophrenia, MOP receptor mediated G-proteins have a reduced stimulatory activity compared to membrane preparations taken from control animals. The observed distinct changes of opioid receptor functions in different areas of the brain do not explain the augmented nociceptive threshold described in these animals.

Keywords: G-protein stimulation, [^{35}S]GTPγS binding, brain membranes, opioid receptors, DAMGO, schizophrenia animal model

1. INTRODUCTION

Schizophrenia is a chronic neuropsychiatric disorder affecting approximately 1% of the population worldwide (for review see: Schultz et al. 2007; Sarkar et al. 2015). It affects equal numbers of men and women, but the onset is often later in women than in men. Schizophrenia is characterized primarily by positive and negative symptoms and cognitive disturbances. Positive symptoms include hallucinations, voices that converse with or about the patient, and delusions that are often paranoid. Negative symptoms include flattened affect, loss of a sense of pleasure, loss of will or drive, and social withdrawal. Medical interventions can control especially the positive signs, but virtually all antipsychotics have neurologic or physical side effects.

Pain is a subjective phenomenon, not fully understood, which is manifested abnormally in most of the neurological disorders including schizophrenia (Antioch et al. 2015). Pain transmission is partially regulated by opioid receptors located in the central and peripheral nervous system. Heterogeneous opioid receptors (MOP, DOP and KOP receptors) are cell membrane integrated proteins belonging to the protein family of G-protein coupled receptors (GPCRs) (Al-Hasani and Bruchas, 2011; Feng et al. 2012; Benyhe et al. 2015; Thompson et al. 2015). GPCRs constitute the largest and physiologically very important membrane protein family that recognizes a variety of environmental stimuli, and are drug targets in the treatment of numerous diseases. Based on clinical studies, schizophrenic patients are often associated with abnormal pain sensitivity (Lévesque et al. 2012; Wojakiewicz et al. 2013). The causes and nature of insensitivity to pain in schizophrenia remain unknown. The role of endorphins and the association of cognitive dysfunction and negative symptoms have recently been postulated (Urban-Kowalczyk et al. 2015).

Developing valuable animal models for complex psychiatric disorders, including schizophrenia, is essential to increase our understanding of the neurobiological basis of the disease and for the development of effective drugs with improved therapeutic efficacy (Marcotte et al. 2001; Jones et al. 2011). Higher pain thresholds in thermal nociception but increased levels of mechanical allodynia have been observed by the use of two neurodevelopmental rat models of schizophrenia (Franěk et al. 2010). All available animal models of this disorder fit into four different induction categories: developmental, drug-induced, lesion or genetic manipulation. Combination of the different methods in obtaining model animals for schizophrenia is more perspective to establish reliable animal model of the disease. Recently a chronic animal model starting from Wistar rat strain that shows some schizophrenia-related deficits by applying selective breeding after subchronic ketamine administration connected with postweaning social isolation (complex treatment) has been developed in our laboratory (Kékesi et al. 2015; Horváth et al. 2015). Distinct changes in central opioid receptor functions (Kékesi et al. 2011) and selective disturbance in pain sensation (Tuboly et al. 2009) have been described in naive animals after social isolation and ketamine treatment (Becker et al. 2003, 2006). The aim of the present study was to investigate and compare the MOP receptor mediated cellular signaling in four brain regions of 'the three hit schizophrenic' model and control animals. Transmembrane signaling was in vitro triggered by the MOP receptor selective synthetic opioid peptide, DAMGO, and monitored by [^{35}S]GTPγS binding experiments.

2. MATERIALS AND METHODS

2.1 Animals

Two experimental groups of 12-12 rats were compared: naive socialized male Wistar rats without ketamine treatment (control group); and the 22nd generation of selectively bred male rats with social isolation and ketamine treatment (model group). All experiments were carried out

with the approval of the Hungarian Ethical Committee for Animal Research (Reference number: XIV/03285/2011) in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. 32.x). The paradigm for selective breeding has previously been described (Petrovszki et al. 2013). Briefly: Wistar rats, after weaning at 3 weeks of age, were tested with the tail-flick (TF) test (48 °C hot water) to assess pain sensitivity and then housed individually for 28 days. The animals were intraperitoneally (i.p.) treated with ketamine (Calypsol, Gedeon Richter Plc., Budapest, Hungary; 30 mg/kg, 4 mL/kg, daily, 5 times/week, 15 injections in total) from 5 to 7 weeks of age. Then the animals were re housed (4-5/cage), and 1 week of recovery followed with no treatment. Starting at the age of 9 weeks, the pain sensitivity with TF test, the sensory gating with prepulse inhibition, and the cognitive functions and stereotypic behavior on hole-board test were assessed. Animals with the highest level of disturbances in these parameters were used for selective breeding throughout 22 generations (Petrovszki et al. 2013; Kékesi et al. 2015).

2.2 Chemicals

Tris-HCl, EGTA, NaCl, $MgCl_2$ x $6H_2O$, GDP, the GTP analogue GTPγS, were purchased from Sigma-Aldrich (Budapest, Hungary). The highly selective MOP receptor agonist enkephalin analogue Tyr-D-Ala-Gly-$_{NMe}$Phe-Gly-ol (DAMGO) was obtained from Bachem Holding AG (Bubendorf, Switzerland). DAMGO was dissolved in water and was stored in 1 mM stock solution at −20 °C. The radiolabeled GTP analogue, [^{35}S]GTPγS (specific activity: 3.7 x 10^{13} Bq/mmol; 1000 Ci/mmol) was purchased from Hartmann Analytic (Braunschweig, Germany). The Ultima Gold™ MV harmless scintillation cocktail was purchased from PerkinElmer (Boston, USA).

2.3 Rat brain membrane homogenate preparation for G-protein activation assays

Control and model rats were decapitated and their brains were quickly removed, dissected on dry ice (prefrontal cortex, thalamus, olfactory bulb and hippocampus), frozen in liquid nitrogen and stored at −80 °C until further processions. The dissected parts of the brains were prepared for obtaining crude membrane fractions according to Benyhe et al, 1997 and used for the

[^{35}S]GTPγS binding experiments. Briefly, the carefully thawed and ice-cooled tissue samples were homogenized on ice in 50 mM Tris-HCl buffer (pH 7.4) using a Teflon-glass homogenizer. The homogenate was centrifuged at 40,000 x g for 20 min at 4 °C and the resulting pellet was resuspended in fresh buffer and incubated for 30 min at 37 °C. The centrifugation step was repeated with the same conditions, and the final pellet was resuspended in ten volume (10 ml buffer/g original tissue) ice-cold TEM buffer (50 mM Tris-HCl, 1 mM EGTA, 5 mM $MgCl_2$, pH 7.4) to give an approximate protein concentration of 3-5 mg/ml. Protein concentrations were measured according to Bradford, 1976. Membrane samples were then aliquoted into Eppendorf tubes containing 40-60 µl membrane suspensions (enough for 24 reaction tubes in the binding assays) and stored at –80 °C for use.

2.4 Functional [^{35}S]GTPγS binding assays

In [^{35}S]GTPγS binding experiments we measured the GDP→GTP exchange of the Gαi/o proteins in the presence of a given ligand to determine the potency of the ligand and the maximal efficacy of the activated G-proteins. The nucleotide exchange is monitored by a radiolabeled, non-hydrolysable GTP analogue, [^{35}S]GTPγS. The functional [^{35}S]GTPγS binding experiments were performed as previously described (Traynor and Nahorski, 1995), with modifications. Briefly the membrane fractions were incubated in 24 polystyrene test tubes (Starstedt Co.) for one series at 30 °C for 60 min in Tris-EGTA buffer (pH 7.4) composed of 50 mM Tris-HCl, 1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, containing 20 MBq/0.05 cm^3 [^{35}S]GTPγS (0.05 nM), around 10 µg/ml membrane protein and increasing concentrations (10^{-10} – 10^{-5} M) of DAMGO. The experiments were performed in the presence of excess GDP (30 µM) in a final volume of 1 ml/reaction tube. Total binding was measured in the absence of test compounds, non-specific binding was determined in the presence of 10 µM unlabeled GTPγS and subtracted from total binding. The difference between total and non-specific binding values represents basal activity. The reaction was terminated by rapid filtration under vacuum (Brandel M24R Cell Harvester), and Whatman GF/B glass fiber filters were washed three times with 5 ml ice-cold 50 mM Tris-HCl (pH 7.4) buffer. The radioactivity of the dried filters was detected in Ultima Gold™ MV aqueous scintillation cocktail with Packard TriCarb 2300TR liquid scintillation counter. [^{35}S]GTPγS binding experiments were performed in triplicates and repeated at least three times.

2.5 Data analysis

Experimental data were presented as means ± S.E.M. Points were fitted with the professional curve fitting program, GraphPad Prism 5.0 (GraphPad Prism Software Inc., San Diego, CA), using non-linear regression analysis. In the [^{35}S]GTPγS binding assays the 'Sigmoid dose-response' fitting was used to establish the maximal stimulation or efficacy (E_{max}) of the receptors G-protein and the ligand potency (EC_{50}). Stimulation was given as percent of the specific [^{35}S]GTPγS binding observed over the basal activity, which was settled as 100%. Unpaired t-tests with two-tailed P-value were performed to determine the significance level, using GraphPad Prism 5.0.

3. RESULTS

In agreement with our recent studies (Petrovszki et al. 2013; Kékesi et al. 2015; Horváth et al. 2015), the selected model rats involved in the in vitro experiments showed decreased acute heat pain sensitivity indicated by longer tail-flick latency compared to the naive (control) rats. Accordingly, tissue samples from these animals were used to determine biochemical changes in the cellular signaling in both groups. The MOP receptor mediated G-protein activation was investigated by [^{35}S]GTPγS binding stimulation assays as suggested by Traynor and Nahorski, 1995. Cellular membranes were prepared from four brain regions of control and 'schizophrenic' model rats: prefrontal cortex, thalamus, olfactory bulb and hippocampus. MOP receptors were activated by the addition of DAMGO (Tyr-D-Ala-Gly-$_{\mathbf{NMe}}$Phe-Gly-ol), a highly selective synthetic enkephalin derivative, first described and used by Handa et al, 1981. Beside morphine, the primary ligand for MOP receptor, the synthetic neuropeptide DAMGO also remained a gold standard in studying MOP receptor functions.

Increasing concentrations of DAMGO produced dose-dependent stimulations of [^{35}S]GTPγS binding in each brain region (Fig. 1). The highest activation of G-proteins was observed in membranes prepared from thalamus of control animals, while the lowest stimulation was found in hippocampal membrane preparations. In olfactory bulb membranes DAMGO mediated stimulation was also prominent and a moderate activation by the peptide was obtained in the prefrontal cortex. The presence of 10 μM naloxone, the general antagonist of multiple opioid receptors, was completely able to inhibit DAMGO mediated stimulations indicating the involvement of opioid receptors in the observed transmembrane signaling processes (data not shown).

MOP receptor mediated activation of G-proteins was consistently lower in membranes prepared from model/treated animals in each brain regions tested (Fig. 1, Table 1). The decrease in G-protein activation is characterized by lower maximal stimulation (E_{max}) values together with a rightward shift of the dose-response curves. Latter is associated with an obvious decrease in the ligand potency (−log EC_{50}) values. Efficacy (maximal stimulation, E_{max}) changes were statistically significant in the case of thalamus (***, P=0.0007; two-tailed P value, t=9.434, df=4) and olfactory bulb (**, P=0.0036; two-tailed P value, t=6.107, df=4). F test to compare variances showed that variances were not significantly different. The observed decrease in E_{max} values in membranes prepared from prefrontal cortex and hippocampus was not significant. Decreases in potency values were seen in each brain regions, but the numeric change was statistically significant only in hippocampal membranes (**, P=0.0021; two-tailed P value, t=7.08, df=4), although the overall G-protein activation was the weakest in this preparation.

4. DISCUSSION

Aberrations of pain experience occur frequently in psychiatric disorders and hence pathological alterations in the basic mechanisms underlying pain experience can be expected. Nevertheless, pain perception, as one of the most important basic mechanisms of pain experience, has rarely been assessed experimentally in psychiatric disorders. Review of clinical and experimental data indicates that in most situations behavioral pain reactivity and self-reported responses to pain are reduced in schizophrenia (de la Fuente-Sandoval et al. 2012; Lévesque et al. 2012; Wojakiewicz et al. 2013). However, there is little or no physiologic or biochemical evidence supporting pain insensitivity in schizophrenia. It can be suggested that the widely accepted notion of reduced pain sensitivity in schizophrenia is related more to a different mode of pain expression than to a real endogenous analgesia. Further studies are required and potential directions for future research are proposed to clarify this issue (Bonnot et al. 2009).

Endogenous analgesia, a likely cause of the decreased pain sensation is expected to be mediated at least partially by opioid receptors (Horváth and Kékesi, 2006; Costantino et al. 2012; Corder et al. 2013). The goal of the present study was therefore to investigate and compare the effectiveness of opioid peptide mediated signal transduction in different brain regions in normal control animals and in a recently developed animal model of schizophrenia (Petrovszki et al. 2013; Horváth et al. 2015; Kékesi et al. 2015). Valuable animal models are important for the investigation of mechanisms and therapeutic approaches in various human neurological disorders. It was hypothesized that in the model animals an enhanced opioid receptor signaling occurs that can be a reason of the decreased pain sensitivity. Transmembrane signaling was studied in vitro by the use of [^{35}S]GTPγS binding experiments in membranes prepared from four different regions of control and model rat brains. This is an accurate, relatively fast and trusty biochemical method to monitor signal transduction events (Traynor and Nahorski, 1994; Sim et al. 1995).

The brain regions used in our study were selected on the basis of their connection to either pain modulation or opioid receptor content. Thus prefrontal cortex has been proposed to control pain perception by modulating cortico-subcortical and cortico-cortical pathways (Lorenz et al. 2003).

The thalamus is one of the important structures that receives projections from multiple ascending pain pathways and thalamic nuclei are involved in the sensory discriminative and affective motivational components of pain (Ab Aziz and Ahmad, 2006; You et al. 2013). Moreover, DAMGO was the most efficacious opioid agonist ligand in membranes prepared from rat thalamus in [^{35}S]GTPγS binding experiments (Selley et al. 1997). The olfactory bulb is the first site for the processing of olfactory information in the brain and its deregulation is often associated with neurodegenerative disorders. Although this tissue is not directly connected to pain perception, a recent proteomic study about the molecular composition of the human olfactory bulb has shown the notable presence of proteins involved in opioid signaling (Fernández-Irigoyen et al. 2012). Hippocampus plays also an active role in suppressing pain especially during times of stress (Ford et al. 2011), and some abnormalities in hippocampal functioning with persistent pain has also been described (Mutso et al. 2012). The presence of MOP receptors in rat hippocampus has been confirmed by autoradiographic (Slamberova et al. 2003) and immunohistochemical (Drake and Milner, 2002) studies.

Although an enhanced or at least not disturbed opioid mediated signaling was expected in the model animals, it was clearly found that G-protein activation via MOP receptors by the synthetic neuropeptide DAMGO was substantially decreased in each brain region studied. The impaired transmembrane signaling is characterized by lower efficacy values (Table 1) combined with a shift to the right of the DAMGO dose-response curves (Fig. 1). Some of the changes were indeed statistically significant. Similar results, i.e. decreased G-protein signaling by either ketamine treatment or social isolation in the full cortex of rat brain and spinal cord, were obtained at an earlier stage of the animal model used in this study (Kékesi et al. 2011). [^{35}S]GTPγS binding was also determined in the prefrontal cortex and the hippocampus in an earlier model of schizophrenia (Becker et al. 2006). Analyzing [^{35}S]GTPγS binding in the frontal cortex, a significant effect was found of housing conditions, suggesting increased efficacy in singly housed rats. Analysis amongst the four experimental groups failed to show significant differences. In the hippocampus, however, the experimental conditions had no effect and [^{35}S]GTPγS binding was not changed as a result of ketamine pretreatment or housing conditions. The authors concluded that [^{35}S]GTPγS binding was unaffected in the hippocampus, whereas it

was increased in the frontal cortex of singly housed rats. Latter observation is in apparent conflict with our results found in the prefrontal cortex, which might be explained by the differences in the models and experimental arrangements used. Moreover, the increased G-protein stimulation in their saline injected single animals was decreased upon ketamine treatment indicating an impairment in G-protein signaling, which agrees with our observations. In further schizophrenia animal models based on phencyclidine (PCP) treatments a significant reduction in [^{35}S]GTPγS binding connected with an increase in the levels of the endocannabinoid 2-arachidonoylglycerol (2-AG) in the prefrontal cortex was reported (Vigano et al. 2009). In the nucleus accumbens and ventral tegmental area the increased endocannabinoid levels were accompanied by unaltered CP55,940-stimulated [^{35}S]GTPγS binding and CB1 cannabinoid receptor expression in PCP treated rats (Seillier et al. 2010). Taken together the decreased or unchanged G-protein activation profile is often present in various brain areas of different animal models of schizophrenia.

The observed inhibition of MOP receptor signaling in our model animals does not support the decreased pain sensitivity found in animal models of schizophrenia and in schizophrenic patients. Such a decrease in MOP receptor mediated transmembrane signaling would have led to hyperalgesia rather than an increased antinociception in the animals. Our results suggest that the decreased pain sensitivity observed in these animals could not be due to enhanced MOP receptor expression or signaling. Further studies are required to clarify the other potential mechanisms (e.g. changes in DOP or adrenergic, serotoninergic, cannabinoid receptor functions) of the hypoalgesia and the causes of decreased MOP receptor functions, e.g. the new substrain might have enhanced level of endogenous opioid ligands leading to MOP receptor internalization.

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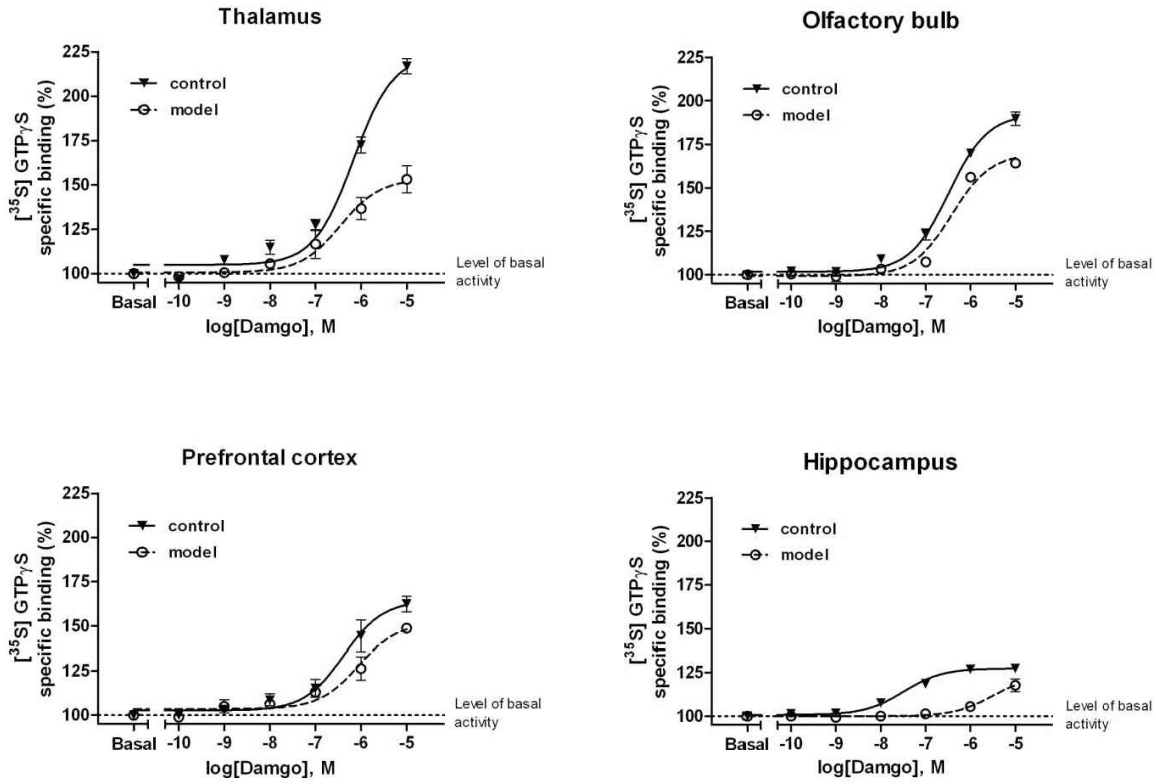

Fig. 1

MOP receptor signaling mediated by DAMGO in membranes prepared from four brain regions of control and model rats.

Percent increases (%) in the specifically bound radiolabeled nucleotide [^{35}S]GTPγS are given over the basal (taken as 100%) activity as a function of increasing DAMGO concentrations (10^{-10} – 10^{-5} M). Points represent mean values ±S.E.M. for three experiments performed in triplicate. „Basal" indicates constitutive G-protein activity level in the absence of any stimulating ligand.

Table 1

G-protein activation by the MOP receptor agonist DAMGO in different regional brain membrane preparations of control and model rats

Brain region in obtaining cell membranes	Maximal stimulation (efficacy) $E_{max} \pm$ S.E.M. (%)		DAMGO Potency Log $EC_{50} \pm$ S.E.M.	
	Control	Model	Control	Model
Thalamus	223.3 ± 5.8	153.3 ± 4.6***	-6.170 ± 0.094	-6.459 ± 0.204^{NS}
Olfactory bulb	192.4 ± 2.3	169.7 ± 2.9**	-6.489 ± 0.059	-6.424 ± 0.089^{NS}
Prefrontal cortex	164.3 ± 3.9	152.1 ± 3.7^{NS}	-6.384 ± 0.144	-6.052 ± 0.151^{NS}
Hippocampus	127.5 ± 0.8	123.3 ± 4.4^{NS}	-7.491 ± 0.101	-5.509 ± 0.261**

Experimental data were processed by GrapPad Prism 5.0 using the sigmoid fit option of the dose-response curves. NS: not significant; **<0.01; ***, P<0.001 based on unpaired t-tests.

CONTRIBUTORS

All authors contributed to and have approved the final manuscript.

Sandor BENYHE and Gyöngyi HORVÁTH designed the study

Sandor BENYHE wrote the manuscript

Gyöngyi HORVÁTH designed the animal model

Alexandra BÜKI and Gabriella KÉKESI made and maintained the animal models and prepared the brain samples

Edina SZŰCS made the membrane preparations and done the biochemical experiments. She also made the data processing including statistical analysis

Conflict of interest

Hereby ALL authors disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three (3) years of beginning the work submitted that could inappropriately influence, or be perceived to influence, their work.

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Figure(s)
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