A selective metabotropic glutamate receptor 7 agonist: Activation of receptor signaling via an allosteric site modulates stress parameters in vivo

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Metabotropic glutamate receptor (mGluR) subtypes (mGluR1 to mGluR8) act as important pre- and postsynaptic regulators of neurotransmission in the CNS. These receptors consist of two domains, an extracellular domain containing the orthosteric agonist binding site and a transmembrane heptahelical domain involved in G protein coupling (1). Pharmacological studies for group III mGluRs are most frequently performed with L-2-amino-4-phosphonobutyrate (L-AP4), which directly activates glutamate receptors in the transmembrane region of mGluR7, and we demonstrate that this allosteric agonist has little, if any, effect on the potency of orthosteric ligands. Here we provide evidence for full agonist activity mediated by the heptahelical domain of family 3 G protein-coupled receptors (which have mGluR-like structure) that may lead to drug development opportunities. Further, AMN082 is orally active, penetrates the blood–brain barrier, and elevates the plasma stress hormones corticosterone and corticotropin in an mGluR7-dependent fashion. Therefore, AMN082 is a valuable tool for unraveling the role of mGluR7 in stress-related CNS disorders.

Materials and Methods

Stable Cell Lines. Generation, culture, and pharmacological characterization of stable cell lines for mGluR1b, mGluR2, mGluR3, mGluR4, mGluR5a, mGluR6, mGluR7a, mGluR7b, mGluR8a, N-methyl-D-aspartate receptor (NMDAR) 1a/2A, NMDAR1a/2B, and GluR3i have been described (10, 15–18).

Conflict of interest statement: The authors are employees of Novartis Pharma AG, which is interested in developing glutamatergic compounds for stress-related disorders.

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Abbreviations: mGluR, metabotropic glutamate receptor; L-AP4, L-2-amino-4-phosphonobutyrate; AMN082, N,N′-dibenzhydryl-1,2-diamine dihydrochloride; ACTH, corticotropin; NMDAR, N-methyl-D-aspartate receptor; L-503, L-serine-O-phosphate.

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Construction of Chimeric Receptors and Corresponding Cell Lines. cDNAs encoding wild-type mGluR6 and mGluR7b were described previously (16, 19). cDNAs encoding chimeric mGluR6/7b and mGluR7/6 proteins were constructed by using the PCR overlap extension approach (3); the mGluR6/7b construct contains 570 aa derived from the N-terminal extracellular region of mGluR6 and the remaining C-terminal portion of mGluR7b, comprising the entire transmembrane region; mGluR7/6 is essentially the reverse chimera with the fusion point at amino acid 575. The four constructs were used to generate stably transfected cell lines by means of Flp recombinase-mediated integration (kit from Invitrogen) in CHO-C4 cells (Novartis Pharma AG, Basel), which contain one specific Flp recognition target (FRT) site; the identical host was used for all of the four new cell lines.

GTPγ35S Binding Assays. Membranes were prepared from transfected mGluR2-, mGluR3-, mGluR4-, mGluR6-, mGluR7a-, mGluR7b-, mGluR8a-, mGluR6/7b-, and mGluR7/6-expressing cells, and GTPγ35S binding assays were conducted by using the protocol described by Maj et al. (10).

Second-Messenger Assays. Measurements of cAMP accumulation were performed as previously described by using CHO cell lines stably expressing individual mGluR subtypes (15, 16). Measurement of [3H]inositol phosphate formation was done according to Gasparini et al. (18), and calcium measurements were done as described by Maj et al. (10).

[3H]LY341495 Binding Assay. Membrane fractions of CHO cells stably expressing mGluR7a (see above) were diluted in assay buffer (10 mM KH2PO4/100 mM KBr, pH 7.6), homogenized briefly by using a Polytron homogenizer (IKA Labortechnik, Staufen, Germany), and incubated for 10 min at 30°C. Assay mixtures were prepared in 96-well microtiter plates. The composition of the assay mixtures in a final volume of 200 µl per well was as follows: 10 mM KH2PO4/100 mM KBr (pH 7.6), 50 µg of pretreated membrane protein (IKA Labortechnik), 1.5 mg of wheat germ agglutinin scintillation proximity assay (WGA SPA) beads (Amersham Biosciences), 10 nM [3H]LY341495, and the test compounds at the appropriate concentrations. Nonspecific binding was measured in the presence of 1 mM l-serine-O-phosphate (L-SOP). The samples were incubated for 60 min at room temperature (with shaking), before being counted in a TopCount (Packard). Data were analyzed by using nonlinear regression in the PRISM program (GraphPad, San Diego). IC50 values were converted into Ki values by using the Cheng and Prusoff equation (20).

Animal Procedures, in Vivo AMN082 Administration. mGluR7−/− mice were generated as described in ref. 21 from E14 (129/Ola) embryonic stem cells. All of the mice in the studies reported here carried wild-type or mutant mGluR7 alleles on a 14th-generation (F14) C57BL/6 genetic background. Age-matched groups of mGluR7−/− and mGluR7+/+ mice were generated as described (14). Male animals were used in all experiments. Food pellets and tap water were available ad libitum. Male mGluR7−/− and littermate mGluR7+/+ mice were injected orally (p.o.) with vehicle or 1–6 mg/kg AMN082. One hour later, mice (mGluR7−/− and mGluR7+/+ mice in a randomized order) were decapitated rapidly (within 30 sec after first touching the cage), and trunk blood was collected (n ≥ 9 per genotype). All animal experiments were subject to institutional review and conducted in accordance with the Veterinary Authority of Basel-Stadt.

Hormone Measurements. Plasma corticosterone and ACTH concentrations were measured by using commercially available RIA kits (ICN) as described (14). The inter- and intraassay coeffi-
agonist activity of L-glutamate (set to 100%; Fig. 1C). The stimulating effects of AMN082 were almost additive with those of L-glutamate and DL-AP4 (238 ± 11% and 336 ± 19%, respectively), whereas DL-AP4 plus L-glutamate (both at maximally active concentrations) produced a smaller stimulation than DL-AP4 alone (133 ± 4% vs. 216 ± 12%, Fig. 1C). Thus, L-glutamate and DL-AP4 are likely to interact at the same receptor site, and the activity of the full agonist DL-AP4 seems to be inhibited by the partial agonist L-glutamate. Next, the group III mGLR-selective antagonists MSOP and CPPG were tested against concentration–response curves of AMN082 conducted in the presence of submaximal DL-AP4 (Fig. 1D): the DL-AP4 component was completely abolished by the antagonists, whereas there was no inhibition of the AMN082-stimulated GTPγS binding. Together, the data of Fig. 1C and D suggest that AMN082 activates mGLR7 signaling most likely by binding to a different site than the orthosteric ligands L-glutamate, DL-AP4, MSOP, and CPPG.

Concentration–response curves for AMN082, DL-AP4, and L-glutamate are compared in Fig. 2A. AMN082 is far more potent than the orthosteric ligands, DL-AP4 and L-glutamate; the EC50 values (95% confidence intervals) are 260 nM (200; 360), 540 μM (440; 670), and 700 μM (580; 850), respectively. To analyze a potential cooperativity between L-glutamate site ligands and AMN082, concentration–response curves for AMN082 at different fixed concentrations of L-glutamate and, inversely, curves for L-glutamate vs. fixed concentrations of AMN082 were conducted (Fig. 2B–D). The EC50 of AMN082 varied between 140 and 290 nM with largely overlapping 95% confidence intervals (Fig. 2B and D). Similarly, the EC50 of L-glutamate was consistently between 640 and 830 μM, irrespective of the added concentration of AMN082 (Fig. 2C and D). To address whether binding of AMN082 to mGLR7 affects the binding affinity of ligands for the L-glutamate site, we analyzed displacement of 10 nM [3H]LY341495 (a competitive mGLR antagonist) binding from membranes prepared from CHO cells stably expressing mGLR7b; up to 30 μM AMN082 showed no displacement of this radioligand. In contrast, 10 mM L-AP4, L-glutamate, or L-SOP abolished 100% of specific binding (data not shown).

AMN082 Directly Interacts with the Heptahelical Region of mGLR7.

Next, we intended to localize the binding site of AMN082 to one discrete region of the mGLR7 protein and decided to use
constricts of wild-type mGluR7b and mGluR6 as well as two chimeras: the mGluR6/7b construct contains the N-terminal extracellular region of mGluR6 and the C-terminal portion of mGluR7b comprising the entire transmembrane region; mGluR7/6 is the reverse chimera (see Materials and Methods and Fig. 3). When using the stimulation of GTPγS binding, the activity of AMN082 on mGluR6/7b and mGluR7/6 chimeras was very similar to wild-type mGluR7b and mGluR6, respectively: AMN082 stimulated mGluR7b and mGluR6/7b by 150–200% relative to the maximal effect of DL-AP4, but AMN082 produced only minor effects on mGluR6- and mGluR7/6-expressing membranes (10–25% relative to maximal DL-AP4 stimulation) (Fig. 3). It is interesting to note that the stimulating effects of AMN082 in combination with DL-AP4 were more than just additive on those mGluR7-expressing, but not on mGluR6/7b-expressing, membranes (Fig. 3A and C). In addition, initial attempts using truncated mutants with deleted extracellular domains were made, but no activation with DL-AP4 or AMN082 was observed (data not shown); it cannot be ruled out that their translated proteins were misfolded or incorrectly inserted into membranes.

Selectivity Profiling of AMN082. Before we addressed the activity of AMN082 at all eight known mGluRs and at three selected ionotropic receptors, we confirmed that there was no significant binding interaction of 1 μM AMN082 with 30 different nervous system targets using a radioligand displacement assay; this list included a selection of receptors for adrenaline, dopamine, GABA, histamine, acetylcholine, opiates, serotonin, and substance P plus selected neurotransmitter reuptake sites (n = 2–4 determinations per target; data not shown). Fig. 4 shows the effects of AMN082 on all eight mGluRs and on three ionotropic GluRs. The activating effect of 3 μM and 10 μM AMN082 was selectively seen at mGluR7a and mGluR7b with large efficacies of 70–140% (relative to maximal DL-AP4 effects) when using the stimulation of GTPγS binding (Fig. 4A and D). Under an identical assay design, AMN082 (up to 10 μM) elicited little or no stimulating effects on membranes from mGluR2-, mGluR3-, mGluR4-, mGluR6-, or mGluR8a-expressing cells, and there was also no activation of GTPγS binding in untransfected CHO cells (Fig. 4B and D). Measurements of phosphoinositidol hydrolysis were done to address whether AMN082 activates group I mGluR subtypes. AMN082 displays neither agonist-like nor positive modulatory activity at mGluR1b- or mGluR5a-expressing cells (Fig. 4D), Functional agonist and modulatory activities of AMN082 were also excluded for two NMDAR subtypes plus one α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subtype (NMDAR1α/2A, NMDAR1α/2B, and GluR3; Fig. 4D); this test was done via cytoplasmic calcium determinations by using stably transfected cell lines. Furthermore, up to 10 μM, AMN082 showed no antagonist-like effects at any of the tested mGluR or ionotropic GluR subtypes when using the functional receptor assay formats described above (Fig. 4).

In Vivo Activity of AMN082: Modulation of Stress Hormones in an mGluR7-Dependent Fashion. Unlike all known l-glutamate site ligands for group III mGluRs, AMN082 readily passes the
AMN082 results in 0.29 nM level to deficient mice (mGluR7a wild-type mouse strain (C57BL/6)) increases plasma corticosterone in a dose-dependent manner in AMN082. This compound acts via a previously undescribed site, In these studies, we present the first selective mGluR7 agonist, Discussion (Fig. 5).

Administration in wild-type animals, but not in mGluR7-deficient mice (Fig. 5A). AMN082 (6 mg/kg administered p.o.) increased plasma corticosterone level in naïve C57BL/6 mice. (C) AMN082 increased plasma ACTH level in mGluR7−/− mice to the same relative level as that shown for corticosterone, but no change was observed in mGluR7+/− mice. Data were normalized to each vehicle control and represent the mean ± SEM from 9–13 individual mice per group. **, P < 0.01 vs. vehicle control; two-way ANOVA followed by Fisher’s post hoc test.

Fig. 5. AMN082 induces stress hormone increases in an mGluR7-dependent fashion. Vehicle or AMN082 was administered to mice orally (p.o.), and 1 hour later mice were decapitated and blood was collected. Plasma corticosterone and ACTH levels were measured with RIA kits. (A) Effect of 1 and 6 mg/kg AMN082 administered p.o. on plasma corticosterone level in naïve C57BL/6 mice. (B) AMN082 (6 mg/kg administered p.o.) increased plasma corticosterone level to ~200% of vehicle control in mGluR7−/−, but not in mGluR7+/−, mice. (C) AMN082 increased plasma ACTH level in mGluR7−/− mice to the same relative level as that shown for corticosterone, but no change was observed in mGluR7+/− mice. Data were normalized to each vehicle control and represent the mean ± SEM from 9–13 individual mice per group. **, P < 0.01 vs. vehicle control; two-way ANOVA followed by Fisher’s post hoc test.

blood–brain barrier upon oral administration: 10 mg/kg oral AMN082 results in 0.29 μmol/kg in total brain tissue, and 14 mg/kg leads to 0.62 μmol/kg in rats and mice, respectively, 1 hour after oral administration (data not shown).

The role of mGluR7 in stress-related behavior is well documented (12–14). Therefore, we analyzed the effect of oral administration of AMN082 on serum levels of the stress hormones corticosterone and ACTH. Fig. 5A shows that AMN082 increases plasma corticosterone in a dose-dependent manner in a wild-type mouse strain (C57BL/6). Next, we used mGluR7-deficient mice (mGluR7−/−) and their wild-type littermates (mGluR7+/+/). Again, oral administration of 6 mg/kg AMN082 elicited an increase of ~200% of plasma corticosterone in mGluR7−/− animals, but no such rise was observed in mGluR7+/− mice (Fig. 5B). Similarly, blood levels of ACTH were also increased to 200% 1 hour after oral AMN082 administration in wild-type animals, but not in mGluR7-deficient mice (Fig. 5C).

Discussion

In these studies, we present the first selective mGluR7 agonist, AMN082. This compound acts via a previously undescribed site, is orally active, and selectively modulates the in vivo levels of two stress hormones, corticosterone and ACTH in wild-type, but not in mGluR7-deficient, mice, substantiating further a role for mGluR7 in stress physiology.

AMN082 elicits a full agonist response, comparable with L-AP4, in the absence of glutamate site ligands. Our data with chimeric receptors strongly suggest the localization of an allosteric agonist site within the transmembrane domain of mGluR7. Definitive proof of this allosteric site awaits the development of an appropriate radioligand, which will enable determination of physicochemical binding characteristics, such as Kd, Kon, and Koff. However, our chimeric receptor data, together with the absence of AMN082 activity at other G-protein-coupled mGluRs expressed in identical host cells, as were used for mGluR7 functional studies, strongly argue that the agonist activity of AMN082 results from a direct compound interaction with the mGluR7 protein. In particular, this mGluR7-dependent agonist activity is seen in both the G protein assay (GTPγS binding) and in second-messenger determinations (cAMP accumulation), which drastically reduces the possibility of AMN082 interacting with components of the intracellular signaling cascades. In GTPγS binding, when using our previously described cell line for human mGluR7b (10, 16–18), the activities of AMN082 and l-glutamate site ligands were close to additive (Figs. 1 and 2). However, AMN082 and DL-AP4 activities were significantly greater than additive on membranes from the new human mGluR7b cell line prepared for the present chimera studies (Fig. 3); significantly greater-than-additive effects of AMN082 and DL-AP4 were also observed on mGluR7a-expressing membranes (Fig. 4A), although to a lesser extent. Similarly, the percent activation induced by AMN082 relative to DL-AP4 differs reproducibly and significantly between the three cell lines. Further studies are required to understand these interesting differences. It may be possible that the mGluR7 receptor number in relation to the expression level of endogenous G proteins is quite different between the three cell lines; this possibility may potentially mask allosteric potentiation in one case but allow it in other cases. Moreover, it is unclear at present whether the alternative C termini of mGluR7a vs. mGluR7b could contribute to these differences. Interestingly, AMN082 was not inhibited by competitive glutamate site antagonists, and neither functional potency nor binding affinity of glutamate site agonists was significantly affected by AMN082 (Figs. 1 and 2).

Accordingly, we propose that AMN082 binds to the transmembrane region of mGluR7 and favors directly the formation of the active receptor state, as postulated also for orthosteric agonists by the two-state model of receptor activation; orthosteric agonist binding seems to be required for AMN082 interaction with mGluR7. Binding to the transmembrane region has also been postulated for the GABAB receptor modulator CQP7930, which was found to directly activate the receptor but with low efficacy (22). The mGluR5-positive modulator 3,3-difluorobenzaldimine acted as a full agonist on mGluR5 deleted of its extracellular domain, but this compound did not act as an agonist itself on the nonmutated full-length receptor (23). A more recent mGluR5 positive modulator, CDPPB, displays partial agonist activity on wild-type mGluR5, and quite interestingly this agonist effect cannot be blocked by orthosteric antagonists (24), which is similar to AMN082. In many ways, AMN082 is also similar to ectopic agonists of M1 muscarinic receptors, such as AC-42, which shows 50–70% efficacy relative to full agonists; this molecule is similar to AMN082. In many ways, AMN082 is also similar to mGluR5 positive modulator, CDPPB, displays partial agonist activity on wild-type mGluR5, and quite interestingly this agonist effect cannot be blocked by orthosteric antagonists (24), which is similar to AMN082. In many ways, AMN082 is also similar to ectopic agonists of M1 muscarinic receptors, such as AC-42, which shows 50–70% efficacy relative to full agonists; this molecule is similar to AMN082.

To our knowledge, the present study of AMN082 provides the first evidence for full agonist activity mediated by the heptahelical domain of a wild-type family 3 G protein-coupled receptor.

Hereofore, the synthesis of subtype-selective group III mGluR agonists that significantly penetrate the blood–brain barrier has been unattainable, presumably because orthosteric receptor activation requires compounds with an α-amino acid moiety and a distal ionizable α-phosphono group, which makes such molecules too hydrophilic for passing biological membranes. In contrast, the allosteric agonist AMN082 structurally is completely unrelated to amino acids. Its physicochemical properties allow oral administration, resulting in significant penetration of the blood–brain barrier, which may lead to drug development opportunities for harnessing pharmacological activity at its novel binding site.
A direct compound interaction of AMN082 with mGluR7 to elicit physiological efficacy in vivo is apparent because AMN082 elevates the plasma levels of the stress hormones corticosterone and ACTH in an mGluR7-dependent fashion; i.e., it does not occur in mGluR7-deficient mice (mGluR7/−/−).

Importantly, 6 min of swim stress elevated corticosterone and ACTH in both mGluR7+/+ (wild-type) and mGluR7/−/− mice, to 400–600%, demonstrating that mGluR7-deficient mice are capable of rapid and robust stress hormone up-regulation, just like wild-type mice (14). However, the elevating effect of AMN082 on stress hormone levels is seen only in mice carrying the functional mGluR7 gene, and it is seen with oral doses that are likely to produce AMN082 brain levels, which are at EC50 or above (see Results). L-glutamate has been implicated as a critical neurotransmitter in the regulation of neuroendocrine functions (27, 28). Whereas a component of glutamatergic control of neuroendocrine function is clearly ionotropic receptor-mediated (29–32), another component seems to act via the G protein-coupled glutamate receptor family, because the nonselective mGluR agonist (1S,3R)-1-amino-cyclopropane-1,3-dicarboxylate (ACPD) when administered intracerebroventricularly (i.c.v.) induced a significant increase in plasma corticosterone (33). An involvement of group I and possibly group II mGluRs has been reported (34–36). It also has been demonstrated that i.c.v. administration of the nonselective group III mGluR agonists L-AP4 and L-SOP activates the hypothalamic-pituitary-adrenal axis (34). Our current data with AMN082 suggest that mGluR7, at least in part, modulates this increase in stress hormones. The mechanism underlying this increase is currently unclear. Johnson et al. (34) suggested, based on an earlier model by Tasker et al. (37), that as group III mGluRs regulate the activity of GABA interneurons in the hypothalamus (38), agonists such as L-AP4 and L-SOP might act by decreasing L-glutamate release from hippocampal-technic tracts. Thus, there would be a decreased tone driving GABAergic interneurons and a disinhibition on ACTH release factor-containing paraventricular nucleus (PVN) neurons of the hypothalamus. Alternatively, they speculate that group III agonists might be acting directly on presynaptic terminals of the GABAergic interneurons and activating heteroautoreceptors, and thus group III mGluR stimulation could directly limit the degree of inhibitory tone that is on the PVN cells. Both scenarios might explain the ability of AMN082 to induce a rise in serum corticosterone and possibly ACTH via mGluR7 activation, as has been seen in the present work.

In conclusion, we have identified a selective mGluR7 agonist, AMN082, that acts via an allosteric site and can serve as an invaluable tool for further unraveling the role of mGluR7 in stress-related CNS disorders.

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