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# Cloning and characterization of the ionotropic GABA receptor subunit ρ1 from pig (*Sus scrofa*)

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## HIGHLIGHTS

- Oocytes injected with retina mRNA generated slow desensitizing GABA currents.
- A cDNA clone was obtained by RT-PCR and cRNA generated from it.
- Xoocytes injected with cRNA were used for pharmacological characterization.
- The cloned nucleotidic sequence is similar to the human transcription variant 2.
- A 2nd splicing variant was cloned with a stop codon early in the reading frame.

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## ABSTRACT

Since human and pig eyes have remarkably anatomical and physiological similitudes swine models have been broadly used for functional studies and therapeutic research. Recently, a GABAρ-mediated relaxation of retinal vascularity suggested that GABAρ signaling may be used to improve retinal blood flow in vascular-driven impaired vision, and a further molecular characterization of GABAρ receptors would be beneficial. However, none of the GABAρ type subunits from pigs has been yet cloned; Among the 19 subunits that compose the family of $GABA_A$ receptors, ρ1–3 subunits are capable of forming homomeric channels. These homomeric receptors are particularly interesting because their pharmacological and kinetic properties are notably different from receptors composed by other $GABA_A$ subunits. Here we report the cloning of the GABAρ1subunit from the pig and the functional expression of homomeric channels in *Xenopus* oocytes. The most notable difference found in the pig GABAρ1 receptor was the absence of a stretch of 17 amino acids near the amino terminus (R41–V58) conserved in the rat and the human. This sequence has a higher nucleotidic match with the transcript variant 2 of the human GABAρ1 subunit. *Xenopus* oocytes injected with cRNA from the receptor generated currents when exposed to GABA that shared all the characteristics of other GABAρ1 subunits in mammals, including its modulation by dopamine. This study will help to increase the knowledge of the genetics of the pig, further the understanding of this important neurotransmitter receptor family and will shed some light in the evolution of these genes among mammals.



## 1. Introduction

Human and pig eyes have remarkably anatomical and physiological similitudes [7,8]; therefore, swine models are preferable to murine models for ontogeny studies and therapeutic research. Recently it was shown that γ-aminobutyrate (GABA) participates in the relaxation of retinal arterioles through the activation of GABAρ type receptors [3], thus, these receptors could be an important target for drugs aimed to improve retinal blood flow in vascular-driven impaired vision.

GABA signaling in the retina, and in the central nervous systems of mammals, is mediated by changes of membrane permeability for chloride and bicarbonate ions upon binding of GABA to ionotropic $GABA_A$ receptors present in cellular membranes. Some of GABA-elicited responses, as those in postsynaptic neurons, activate and desensitize very fast resulting in what is known as "phasic" inhibition [6]; also, low GABA concentrations in the extracellular space can result in the persistent activation of GABA receptors generating a "tonic" inhibition of excitable cells [6]. $GABA_A$ receptors belong to a family of proteins that assemble to form integral ligand gated ion channels (LGICs). Thus far, 19 subunits of this family have been characterized in mammals. The subunits have been

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classified in basis of their sequence identity in the subfamilies α, β, γ, δ, ε, θ, π, and ρ. Most subunits within the $GABA_A$ family share at least 20–50% genomic sequence, while in subunits within a subfamily the identity is at least 70% [14]. $GABA_A$ receptors can be formed by the combination of different subunits with the major adult isoform comprised of 2 α subunits, 2 β subunits and 1 γ subunit [20]. There is strong evidence indicating that the 3 subunits of the ρ subfamily are able to form homomeric functional channels, and may form heteromers with subunits from other subfamilies [14]. Homomeric GABAρ receptors display different pharmacological properties: they are insensitive to bicuculine and to modulation by benzodiazepines [5], are modulated by dopamine and serotonin [12], but most notably, show a slow desensitization after prolonged exposure to the agonist. They were considered a separate type ($GABA_C$) for many years, but recently they are regarded as part of the $GABA_A$ type [13].

Despite the wide use of swine models to study retinal physiology and the important role of GABAρ receptors in retina function, none of the putative GABAρ type subunits from pigs has been yet cloned and the pharmacological correspondence between human and pig GABAρ receptors can only be inferred. Here we used information from the International Swine Genome Sequencing Consortium (SGSC) [2] to clone the pig GABAρ1 receptor subunit. This clone will aid in the molecular characterization of its pharmacological properties and to gain further insight into this family of receptors in mammals.

## 2. Materials and methods

### 2.1. Functional expression

Retina from pig was obtained immediately after death from a local slaughterhouse. The collected tissue was snap frozen in dry ice until further use. mRNA was isolated using the Fast Track mRNA isolation kit from Invitrogen. Half of the recovered mRNA was used for microinjections into *Xenopus* oocytes and after 2–4 days oocytes were tested for expression of neurotransmitter receptors [15], briefly, oocytes were impaled with two microelectrodes filled with 3 M KCl and voltage clamped at −80 mV using a two electrode voltage-clamp amplifier. Oocytes were continuously perfused with gravity-driven frog Ringer's solution [115 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 5 mM Hepes (pH 7.4)] at room temperature (19–21 °C). Data acquisition was performed using WinWCP V 3.9.4 (John Dempster, Glasgow, United Kingdom). The cDNA clone was used as template to generate cRNA as described previously [17] and injected, at a concentration of 0.1 mg/ml, into *Xenopus* oocytes for further pharmacological analysis.

### 2.2. Data analysis

Concentration/response curve for GABA was built as previously reported [10]. Briefly, a logistic equation of the form $I(x) = I_{min} + (I_{max} - I_{min})/[1 + (x/EC50)k]$, where $x$ is the concentration of GABA (in M), $I$ is the amplitude of the GABA-induced current (in nA), and $k$ is the slope of the curve, was fitted to the experimental data (SigmaPlot 10). Experimental data are shown as mean ± SD unless otherwise stated. The rise to maximum was measured between the 10–90% of the ion current activation using clampfit 10.2 (Axon instruments).

### 2.3. cDNA cloning.

After the recent completion of the draft sequencing of the pig genome by the SGSC [2], many ESTs have been reported and are now available for molecular biology analyses [21]. We proceeded to clone the putative pig GABAρ1 receptor subunit identified by an Ensembl automatic analysis pipeline and submitted to UniProtKB under the primary accession number F1S0D1. For this, the remainder of isolated retinal mRNA was reverse-transcripted into cDNA as previously described [16] after confirmation of the presence of transcripts capable of generating functional channels was established by electrophysiology. Primers (Fwd atgttggctgtccagaa; Rev ttatgagaaaatcgacc) were designed using the predicted sequence for the gene reported in the gene bank (NCBI Reference Sequence: XM_001927005.1).

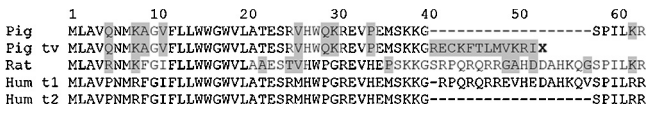


**Fig. 1.** Amino terminus of the ρ1 subunit. A stretch of 17 amino acids in the human transcript variant 1 (Hum t1) near the amino terminus is missing in the pig ρ1 subunit (18 in the rat), but the human transcript variant 2 (Hum t2) share the same amino acidic gap. Overall, the sequence is highly conserved between the three species. The shaded letters highlights the differences with the human sequence. A second transcript variant was cloned from the pig retina (Pig tv) that shared the same splicing site (G40) but the insertion introduced a stop codon after 12 amino acids.

## 3. Results

### 3.1. Electrophysiological findings and cloning

Oocytes injected with mRNA isolated from pig retina elicited non-desensitizing GABA-induced responses of 8.0 ± 5.3 nA ($n$ = 4 oocytes; mean ± SD, data not shown) and with kinetic characteristics similar as GABAρ currents from bovine retina [15]. Confirming our previous results in non-injected oocytes where Western blots did not detected endogenous GABAρ receptors [9], non-injected oocytes also did not respond to GABA even at concentrations as a high as 1 mM [9]. Therefore, the GABA responses in oocytes injected with pig retina mRNA confirmed the presence of full length transcripts for the GABAρ subunit in the mRNA, and cDNA cloning was performed next.

After RT-PCR, a single band of about 1400 bp was obtained and introduced in a vector by T cloning. A sequence of 1389 base pairs with a reading frame of 462 amino acids was confirmed by sequencing. The sequence shared nucleotidic and amino acidic homologies of 91% and 92% with the human and 89% and 91% with the rat (Fig. 1). Three nucleotide substitutions were identified from the predicted sequence (184T > C, 567A > G and 1143C > T, Fig. 2); however, the reported amino acidic sequence was not altered by these nucleotide changes.

### 3.2. cDNA clone pharmacological characterization

Oocytes injected with synthetic mRNA generated from the cDNA clone of the pig retina, expressed membrane receptors, highly sensitive for GABA, with an $EC_{50}$ of 698 ± 489 nM and a Hill coefficient of 2.6 ± 0.2 (mean ± sd; $n$ = 5; Fig. 3). GABA currents rate of activation accelerated with the concentration, from a rise to maximum of 67.8 ± 14.2 s at 0.3 μM GABA to 4.5 ± 1.5 s at 3 μM

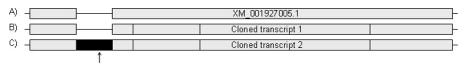


**Fig. 2.** Schematic gene structure of predicted (A) and cloned sequences (B and C). (A) Gene bank sequence XM_001927005.1, predicted GABA ρ1 subunit from the pig. (B) Cloned sequence, functionally active. Vertical lines represent the 3 nucleotidic substitutions found. (C) Transcript variant. Black rectangle illustrates a 140 bp insertion that introduces an early stop codon (arrow).