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Positive Allosteric Modulation of the Muscarinic M_1 Receptor Improves Efficacy of Antipsychotics in Mouse Glutamatergic Deficit Models of Behavior[S]

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ABSTRACT

Current antipsychotics are effective in treating the positive symptoms associated with schizophrenia, but they remain suboptimal in targeting cognitive dysfunction. Recent studies have suggested that positive allosteric modulation of the M_1 muscarinic acetylcholine receptor (mAChR) may provide a novel means of improving cognition. However, very little is known about the potential of combination therapies in extending coverage across schizophrenic symptom domains. This study investigated the effect of the M_1 mAChR positive allosteric modulator BQCA [1-(4-methoxybenzyl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid], alone or in combination with haloperidol (a first-generation antipsychotic), clozapine (a second-generation atypical antipsychotic), or aripiprazole (a third-generation atypical antipsychotic), in reversing deficits in sensorimotor gating and spatial memory induced by the *N*-methyl-D-aspartate receptor antagonist, MK-801 [(5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine]. Sensorimotor gating and spatial memory induction are two models that represent aspects of schizophrenia modeled in rodents. In prepulse inhibition (an operational measure of sensorimotor gating), BQCA alone had minimal effects but exhibited different levels of efficacy in reversing MK-801–induced prepulse inhibition disruptions when combined with a subeffective dose of each of the three (currently prescribed) antipsychotics. Furthermore, the combined effect of BQCA and clozapine was absent in $M_1^{-/-}$ mice. Interestingly, although BQCA alone had no effect in reversing MK-801–induced memory impairments in a Y-maze spatial test, we observed a reversal upon the combination of BQCA with atypical antipsychotics, but not with haloperidol. These findings provide proof of concept that a judicious combination of existing antipsychotics with a selective M_1 mAChR positive allosteric modulator can extend antipsychotic efficacy in glutamatergic deficit models of behavior.

Introduction

Schizophrenia affects approximately 1% of the population and is characterized by positive, negative, and cognitive symptoms (Pantelis et al., 1997, 1999, 2001; Grube et al., 1998; Harvey et al., 1998; Heinrichs and Zakzanis, 1998; Kandel, 2000; Bora et al., 2009). Current antipsychotics are generally successful in treating the positive symptoms (e.g., hallucinations and delusions) but remain largely ineffective in relieving negative and cognitive symptoms and also possess side effects (Speller et al., 1997; Pantelis and Lambert, 2003; Lieberman et al., 2005; Keefe et al., 2007; Gao et al., 2008; Nasrallah, 2008; Sakurai et al., 2013). Hence, there is an unmet need for newer approaches that could target additional symptom domains and produce fewer side effects.

Existing antipsychotics treat positive schizophrenic symptoms by targeting the dopamine system, particularly the D_2 receptor (Conn et al., 2008; Miyamoto et al., 2012). The broader, poorly treated, negative and cognitive domains involve additional pharmacological targets. Glutamatergic neurotransmission, in particular that mediated by the *N*-methyl-D-aspartate receptor (NMDAR), is potentially one such target (Rujescu et al., 2006; Javitt, 2007; Stone et al., 2007; Gordon, 2010). NMDAR antagonists, such as phencyclidine, produce psychotic-like symptoms in humans (Javitt and

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ABBREVIATIONS: ACh, acetylcholine; ANOVA, analysis of variance; BQCA, 1-(4-methoxybenzyl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid; CHO, Chinese hamster ovary; DMSO, dimethylsulfoxide; mAChR, muscarinic acetylcholine receptor; MK-801, (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine; NMDAR, *N*-methyl-D-aspartate receptor; [^{3}H]NMS, *N*-[^{3}H] methylscopolamine; PAM, positive allosteric modulator; PPI, prepulse inhibition.

Zukin, 1991; Murray, 2002; Moghaddam and Javitt, 2012). Subanesthetic doses of MK-801 [(5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine], a more potent NMDAR antagonist, also induce animal behaviors that resemble aspects of schizophrenia, such as deficits of gating mechanisms and spatial memory (Bubeníková et al., 2005; Long et al., 2006; Bradford et al., 2010; van der Staay et al., 2011; Bubser et al., 2014). Accordingly, the ability of novel compounds to reduce MK-801–induced behaviors is commonly used as part of a battery of tests that assess preclinical utility of investigational molecules as potential antipsychotics (Enomoto et al., 2008; Bubser et al., 2014; Park et al., 2014).

Muscarinic acetylcholine receptors (mAChRs), in particular central M_1 and M_4 mAChRs, are implicated in learning and memory and have been pursued as therapeutic targets for cognitive dysfunction, including in schizophrenia (Langmead et al., 2008; Lieberman et al., 2008). Furthermore, cholinergic and dopaminergic networks can interact with one another (Lester et al., 2010). For example, stimulation of mAChRs in the ventral tegmental area increases dopamine release in the striatum and frontal cortex (Gronier et al., 2000), and knockout of M_1 mAChRs increases striatal dopamine levels (Gerber et al., 2001). Importantly, the cholinergic system also interacts with glutamatergic transmission; M_1 mAChRs modulate NMDAR current in hippocampal neurons (Jones et al., 2008). Of note, xanomeline, an M_1 and M_4 mAChR-preferring agonist, improved psychotic symptoms in patients with Alzheimer disease and cognitive function in people with schizophrenia (Bodick et al., 1997; Shekhar et al., 2008). However, further clinical development of xanomeline was precluded due to unacceptable side effects, predominantly reflecting off-target activity via the highly conserved orthosteric site at other mAChR subtypes (Bodick et al., 1997; Wood et al., 1999; Bymaster et al., 2002). Encouragingly, mAChRs possess spatially distinct allosteric sites that offer alternative strategies for greater selectivity (Christopoulos, 2002; Bridges et al., 2010; Bolbecker and Shekhar, 2012; Conn et al., 2014; Kruse et al., 2014; Thal et al., 2016). BQCA [1-(4-methoxybenzyl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid], a highly selective M_1 mAChR positive allosteric modulator (PAM) with no reported off-target activities, reversed scopolamine-disrupted memory, rescued memory deficits in a mouse model of Alzheimer disease, and also had procognitive effects on baseline memory performance (Ma et al., 2009; Shirey et al., 2009; Chambon et al., 2011, 2012; Galloway et al., 2014). The effects may be due to activation of neurons in the prefrontal cortex and hippocampus, as these regions express high levels of M_1 mAChRs (Porter et al., 2002; Ma et al., 2009).

The superior efficacy of existing atypical antipsychotics may be related to their complex polypharmacology (Miyamoto et al., 2005). However, less is known about the potential of combination therapies for improved treatment of negative and cognitive schizophrenic domains. Most atypical antipsychotics have limited affinity for the M_1 mAChR; however, a few (e.g., clozapine and olanzapine) are antagonists at the M_1 mAChR, an action linked to acute memory impairment (Bymaster et al., 1999; Didriksen et al., 2006; Mutlu et al., 2011; Miyamoto et al., 2012). Hence, coadministration of a compound that could specifically activate or potentiate the M_1 mAChR together with select current antipsychotics may offer additional therapeutic value in extending antipsychotic efficacy. This study thus aimed to investigate the effects of a combination of BQCA with selected typical and atypical antipsychotics on MK-801–induced behaviors in mice, specifically prepulse inhibition (PPI) disruption and impaired memory in the Y-maze spatial test.

Materials and Methods

Materials. Chinese hamster ovary (CHO) FlpIn cells and Dulbecco's modified Eagle's medium were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was purchased from ThermoTrace (Melbourne, Australia). Hygromycin-B was purchased from Roche (Mannheim, Germany). *N*-[^{3}H] methylscopolamine ([^{3}H]NMS; specific activity, 85 Ci/mmol) and Ultima gold scintillation liquid were purchased from PerkinElmer Life Sciences (Boston, MA). BQCA, clozapine, and aripiprazole were synthesized in-house, and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). FlpIn CHO cells stably expressing the human M_1 mAChR were generated and maintained as described previously (Abdul-Ridha et al., 2014).

In Vitro Competition Binding Assays. FlpIn CHO cells expressing the human M_1 mAChR were plated at 25,000 cells per well in 96-well Isoplates (PerkinElmer Life Sciences). The next day, cells were incubated in a final volume of 100 μl HEPES buffer (10 mM HEPES, 145 mM NaCl, 1 mM $MgSO_4 \cdot 7H_2O$, 10 mM glucose, 5 mM KCl, 2 mM $CaCl_2$, and 1.5 mM $NaHCO_3$, pH 7.4) containing increasing concentrations of a competing cold ligand carbachol (in the absence or presence of 10 μM BQCA) in the presence of 0.1 nM [^{3}H]NMS for 4 hours at 4°C (to avoid potential confounding effects of competing agonist ligands on receptor internalization while ensuring reactions reached equilibrium). Nonspecific binding was defined in the presence of 100 μM atropine. For all experiments, termination of the assay was performed by rapid removal of the radioligand followed by two 100-μl washes with ice-cold 0.9% NaCl buffer. Radioactivity was determined by addition of 100 μl Microscint scintillation liquid (PerkinElmer Life Sciences) to each well and counting in a MicroBeta plate reader (PerkinElmer Life Sciences).

Animals and Drugs. The experiments were performed using 2- to 4-month-old male C57Bl/6J, M_1 mAChR homozygous knockout ($M_1^{-/-}$), and wild-type ($M_1^{+/+}$) mice in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Behavioral testing procedures were approved by the Animal Ethics Committee of the Monash Institute of Pharmaceutical Sciences. The $M_1^{-/-}$ mice, which were backcrossed to C57Bl/6N for more than 11 generations, and both $M_1^{+/+}$ and $M_1^{-/-}$ mice shared the same C57Bl/6N background (Bymaster et al., 2003; Thomsen et al., 2010). They were bred and genotyped at the Monash Institute of Pharmaceutical Sciences (Parkville, Australia). Mice were housed on a reversed light/dark cycle (lights on 7 PM) with ad libitum access to food and water. As outlined in the *Results* and in Table 1, two different routes of administration were used: C57Bl/6J mice received BQCA via subcutaneous injection, as per a previous study in this mouse strain from our laboratory when investigating another class of mAChR PAM (Suratman et al., 2011), and were tested in both PPI and Y-maze behavioral tests. However, prior studies have shown that $M_1^{+/+}$ and $M_1^{-/-}$ mice on a C57Bl/6N substrain background can exhibit a different PPI profile (Matsuo et al., 2010) compared with C57Bl/6J mice. Given the limited number of animals available for these latter studies and our desire to maximize interanimal consistency, we performed new pharmacokinetic studies that revealed that intraperitoneal injection of BQCA (in a solubilized formulation) resulted in higher exposure in this instance (see *Results*). BQCA (1–20 mg/kg) was suspended in a combination of 50% Pharmasolve (Ashland Inc., Bridgewater, NJ) and 1.1% Tween 20 (Sigma-Aldrich, Castle Hill, Australia) in injection water (PLP, Glendenning, Australia) for subcutaneous injection (Suratman et al., 2011), or dissolved in 15% dimethylsulfoxide (DMSO) (Sigma-Aldrich), 2% Tween 20, and 22 mM Tris buffer (pH 8.9) for intraperitoneal injection. Together with BQCA administration (subcutaneously or intraperitoneally), animals received a single intraperitoneal dose of 0.03–0.25 mg/kg haloperidol

TABLE 1
Experimental groups and reports of ANOVA analysis

Behavioral Test	Mouse Strain	No. of Mice	Drugs (Route)	Main Effects and Interactions after ANOVA Analysis		
				Cohort	*F* Value	*P* Value
PPI	C57Bl/6J	Vehicle and MK-801 controls, 15–17; others, 9–10	BQCA (s.c.) + antipsychotics (i.p.)	MK-801 overall	$F_{(1,\ 343)}$ = 315.3	<0.001
				BQCA alone	None	
				BQCA + haloperidol		
				BQCA effect	$F_{(4,\ 174)}$ = 4.3	0.002
				Haloperidol effect	$F_{(1,\ 174)}$ = 45.0	<0.001
				BQCA × haloperidol interaction	$F_{(4,\ 174)}$ = 3.3	0.013
				BQCA + clozapine		
				BQCA effect	$F_{(4,\ 165)}$ = 2.7	0.032
				Clozapine effect	$F_{(1,\ 165)}$ = 31.6	<0.001
				BQCA + aripiprazole		
				BQCA effect	$F_{(4,\ 160)}$ = 2.7	0.031
				Aripiprazole effect	$F_{(1,\ 160)}$ = 24.6	<0.001
				Prepulses × BQCA × Aripiprazole × MK-801 interaction	$F_{(4,\ 320)}$ = 4.5	0.002
Y-maze	C57Bl/6J	10–13	BQCA (s.c.) + antipsychotics (i.p.)	BQCA alone		
				Arm effect	$F_{(1,\ 59)}$ = 17.2	<0.001
				Arm × MK-801 effect	$F_{(1,\ 59)}$ = 9.7	0.003
				BQCA + haloperidol		
				Arm effect	$F_{(1,\ 124)}$ = 8.4	0.005
				Arm × MK-801 effect	$F_{(1,\ 124)}$ = 6.4	0.012
				BQCA + clozapine		
				Arm effect	$F_{(1,\ 123)}$ = 13.4	<0.001
				Arm × MK-801 effect	$F_{(1,\ 123)}$ = 5.1	0.026
				Arm × BQCA effect	$F_{(2,\ 123)}$ = 3.5	0.034
				BQCA + aripiprazole		
				Arm effect	$F_{(1,\ 132)}$ = 18.0	<0.001
				Arm × MK-801 effect	$F_{(1,\ 132)}$ = 8.6	0.004
PPI	$M_1^{+/+}$ mice (C57Bl/6 Ntac background)	Vehicle and MK-801 controls, 15–16; others, 8–9	BQCA (i.p.) + clozapine (i.p.)	$M_1^{+/+}$ mice		
	$M_1^{-/-}$ mice (C57Bl/6 Ntac background)	Vehicle and MK-801 controls, 13–15; others, 8		MK-801 effect	$F_{(1,\ 67)}$ = 29.0	<0.001
				BQCA effect	$F_{(2,\ 67)}$ = 3.2	0.046
				Clozapine effect	$F_{(1,\ 67)}$ = 8.3	0.005
				$M_1^{-/-}$ mice		
				MK-801 effect	$F_{(1,\ 61)}$ = 26.2	<0.001

(Tocris, Bristol, UK) (dissolved in 1% DMSO saline), 0.5–2 mg/kg clozapine, or 2.5 mg/kg aripiprazole (dissolved in 2% Tween 20 saline). Twenty-five minutes later, mice received an intraperitoneal injection of 0.15 or 0.3 mg/kg MK-801 (Sigma-Aldrich) dissolved in 0.1% ascorbic acid saline, and mice were then subjected to PPI or a Y-maze training session (Y_{train}) 20 minutes afterward (see *Results* and figures for timelines). The doses of antipsychotics were specifically chosen to be subeffective in the model under test, based on previous studies (haloperidol; Bast et al., 2000), or pilot studies conducted in-house (clozapine and aripiprazole). Our pilot study showed that 4 mg/kg clozapine, or 20 mg/kg aripiprazole, reversed PPI disruption induced by 0.3 mg/kg MK-801. Haloperidol, at any doses below those that cause catalepsy (i.e., <0.5 mg/kg, above which catalepsy is observed), was ineffective at reversing the MK-801 effect. In addition, all antipsychotic doses used were either lower than (aripiprazole and clozapine) or within (haloperidol) the clinically comparable range with regard to their likely dopamine D_2 receptor occupancies in humans (Kapur et al., 2003; Natesan et al., 2011).

Assessment of BQCA Exposure. BQCA exposure was assessed in three separate pharmacokinetic studies (see *Results*). Separate groups of C57Bl/6J, $M_1^{+/+}$, and $M_1^{-/-}$ behavioral naïve mice were administered BQCA (subcutaneously or intraperitoneally), with antipsychotics (2 mg/kg clozapine, 0.25 mg/kg haloperidol, or 2.5 mg/kg aripiprazole), or vehicle and were euthanized at time points relevant to the collection of behavioral data (i.e., 45, 90, or 180 minutes postdose). Concentrations of BQCA or antipsychotics, in plasma and/or brain homogenate, were determined via ultra-performance liquid chromatography/mass spectrometry. The concentration of BQCA in the brain parenchyma (C_{brain}) was calculated on the basis of the measured concentration in the brain homogenate ($C_{brain\ homogenate}$), after correcting for the contribution of compound contained within the vascular space of brain samples as follows:

$C_{brain} = C_{brain\ homogenate} - C_{brain\ vasculature}$, where $C_{brain\ vasculature}$ is $C_{plasma} \times V_p$.

C_{brain}, $C_{brain\ homogenate}$, and $C_{brain\ vasculature}$ are the concentrations of compound in the brain parenchyma, brain homogenate, and brain vasculature, respectively (in nanograms per gram). C_{plasma} is the concentration of compound in plasma (in nanograms per milliliter). V_p is the brain plasma volume (0.017 μl/g for C57Bl6 mice; Nicolazzo et al., 2010).

The unbound concentration of BQCA ($C_{unbound}$) in the brain parenchyma was then calculated as $C_{total} \times f_u$, using published fraction free concentration (f_u) values for BQCA (i.e., $f_{u,brain} = 0.126$; Gould et al., 2015). Otherwise, the total concentration of antipsychotics (C_{total}) is presented. The data are expressed in nanomolar units assuming a brain density of 1 g/ml.

PPI Test. PPI was performed in a sound-attenuated room using SR-LAB startle chambers (San Diego Instruments, San Diego, CA). Mice subjected to PPI testing received BQCA (1–20 mg/kg) with or without antipsychotics, and then MK-801 (0.3 mg/kg) or its vehicle. Each PPI session lasted for 1 hour and consisted of three startle pulses (p100, p110, and p120) and was also preceded with 6-, 12-, and 18-db prepulses above a 65-db background for each pulse (referred to as pp6, pp12, and pp18, respectively), to provide a more comprehensive characterization of PPI (Yee et al., 2005). In the first PPI study conducted in C57Bl/6J mice, all treatment groups were pseudo-randomized on each testing day, with at least one vehicle– and MK-801–only treated mouse included as a control per PPI testing day. There were 15–17 mice for the vehicle-only or MK-801–only treatment groups, and there were 9–10 mice for all other treatment groups. The second PPI study was conducted in both $M_1^{+/+}$ and $M_1^{-/-}$ mice using the same PPI protocol, with 13–16 mice for the vehicle and MK-801 controls and 8–9 mice for the other treatment groups. In these PPI studies, the drug effect on the overall PPI measured at the three startle pulses (i.e., p100, p110, and p120) was found to be similar; hence, only PPI data analysis obtained from p120 is reported. To simplify data presentation, the average PPI (%) is presented in the figures, and PPI (%) measured at each prepulse (pp6, pp12, and pp18) is detailed in Table 2. There was no change to the startle response in mice, except for those treated with the highest dose (20 mg/kg) of BQCA alone (in the presence of MK-801) (Supplemental Fig. 1). The average PPI data of p100 and p110 are presented in Supplemental Fig. 2.

Y-Maze Test. Mice received BQCA, clozapine, haloperidol, aripiprazole, or a combination of BQCA and an antipsychotic before MK-801 (0.15 mg/kg) injection. Since a lower dose of MK-801 (compared with 0.3 mg/kg used in PPI) was sufficient to disrupt spatial memory formation in the Y-maze, the dose range of BQCA chosen was also reduced to 5–10 mg/kg. The ranges of the antipsychotics were also reduced relative to those used in the PPI studies: haloperidol, 0.03 to 0.06 mg/kg; clozapine, 0.5 to 1 mg/kg; and aripiprazole, 0.125 to 0.25 mg/kg. Higher doses of aripiprazole (i.e., >0.25 mg/kg) were excluded, because mice had significantly lower locomotor activity in both training and testing sessions. Y-maze experiments were conducted in a manner similar to those previously described with 10–13 mice per treatment group (Dellu et al., 1992; Choy et al., 2008; Shipton et al., 2014). In brief, mice were allowed to explore two arms, with the novel arm closed, in a training session (Y_{train}) that lasted for 10 minutes. Two hours later, the same mice were allowed to explore all three arms for 5 minutes in the testing session (Y_{test}). Behavior was recorded and analyzed with video tracking software (Viewer III; Biobserve GmbH, Bonn, Germany), and the time spent in each arm, during the testing session, was used as a measure of the exploratory preferences of mice. A significant difference between novel and familiar arms was considered as a measure of baseline memory function. In a preliminary study, prior to the full Y-maze test, MK-801 disrupted memory function when it was administered before the training session rather than after (data not shown). This finding is consistent with a previous study in which MK-801 disrupted memory in a novel objection recognition test in mice (Nilsson et al., 2007), suggesting that NMDARs blocked by MK-801 disrupt memory acquisition during the training phase. The Y-maze data of mice treated with the same vehicle used to administer MK-801 (i.e., 0.1% ascorbic acid in saline) and a combination of BQCA and antipsychotics are also reported in Supplemental Fig. 3.

Data Analysis. For the in vitro studies, competition binding parameters were determined via nonlinear regression as described previously (Canals et al., 2012). All affinity values were estimated as logarithms, and statistical comparisons between values were performed by *t* tests using GraphPad Prism software (version 6.0; GraphPad Software Inc., La Jolla, CA). Behavioral data were analyzed using IBM SPSS (version 22.0; IBM, Armonk, NY). PPI (%) was analyzed with analysis of variance (ANOVA) with repeated measures of prepulses (pp6, pp12, and pp18), with drug treatments as main factors. Tukey's post hoc test was then used to determine the level of significance at each prepulse and average values of PPI (%) among groups when there was a main drug effect or an interaction. For Y-maze data, the time spent in novel and familiar arms is presented (in seconds). If the overall main arm effect was significant in each cohort, pairwise ANOVA comparisons were performed between the

TABLE 2

PPI measured with prepulses at 6, 12, and 18 db above the background in C57Bl/6J mice, which received BQCA via the subcutaneous route

Data are presented as means ± S.E.M.

Drug	PPI		
	pp6	pp12	pp18
		%	
Vehicle control	23.6 ± 3.4	36.2 ± 3.0	50.3 ± 2.4
MK-801	−3.0 ± 3.2*	15.7 ± 3.6*	17.4 ± 2.5*
+ 1 mg/kg BQCA	1.1 ± 5.1	12.1 ± 4.7	17.5 ± 6.8
+ 3 mg/kg BQCA	0.8 ± 6.2	6.9 ± 5.0	4.6 ± 5.0
+ 10 mg/kg BQCA	1.4 ± 5.7	11.1 ± 6.0	21.4 ± 6.1
+ 20 mg/kg BQCA	−8.0 ± 4.9	6.2 ± 3.9	17.9 ± 2.5
MK-801 + 0.25 mg/kg haloperidol	4.4 ± 2.0	22.1 ± 3.5	37.1 ± 4.4
+ 1 mg/kg BQCA	6.2 ± 3.6	18.8 ± 3.1	35.6 ± 2.9
+ 3 mg/kg BQCA	7.7 ± 2.7	23.9 ± 4.2	37.8 ± 5.5
+ 10 mg/kg BQCA	10.9 ± 2.9	23.7 ± 3.5	36.9 ± 4.4
+ 20 mg/kg BQCA	12.9 ± 2.0	34.4 ± 4.4	57.8 ± 4.7**
MK-801 + 2 mg/kg clozapine	9.4 ± 3.7	25.7 ± 4.6	37.9 ± 6.2
+ 1 mg/kg BQCA	8.4 ± 2.6	16.7 ± 2.8	36.3 ± 4.2
+ 3 mg/kg BQCA	3.7 ± 6.0	12.5 ± 6.8	27.5 ± 4.9
+ 10 mg/kg BQCA	0.2 ± 3.8	22.4 ± 4.4	38.0 ± 4.4***
+ 20 mg/kg BQCA	14.0 ± 8.0	32.6 ± 4.7	56.4 ± 4.9**
MK-801 + 2.5 mg/kg aripiprazole	8.2 ± 3.2	20.7 ± 3.5	27.7 ± 4.3
+ 1 mg/kg BQCA	0.1 ± 3.8	11.9 ± 5.8	22.7 ± 6.9
+ 3 mg/kg BQCA	−3.6 ± 5.2	14.4 ± 4.2	30.3 ± 3.9
+ 10 mg/kg BQCA	10.0 ± 2.9	17.9 ± 3.9	32.0 ± 4.9
+ 20 mg/kg BQCA	3.9 ± 4.5	26.2 ± 5.2	47.0 ± 3.1**

*$P < 0.05$ (versus vehicle control − MK-801 effect); **$P < 0.001$ (versus MK-801 control at the same prepulse); ***$P < 0.05$ (versus MK-801 control at the same prepulse).

novel and familiar arms in each group. Both PPI and Y-maze data analysis were divided into BQCA alone, BQCA plus haloperidol, BQCA plus clozapine, and BQCA plus aripiprazole cohorts and included their vehicle control in the ANOVA analysis. The drug effects and interactions after the ANOVA analysis are reported in Table 1, and post hoc tests are reported in the *Results* and/or indicated in the figures. Data are presented as means ± S.E.M. A value of $P < 0.05$ was considered statistically significant.

Results

BQCA, But Not Typical or Atypical Antipsychotics, Acts as a PAM of Acetylcholine at the M_1 mAChR. BQCA is a highly selective PAM of acetylcholine (ACh) binding and function at the M_1 mAChR (Ma et al., 2009; Canals et al., 2012). Therefore, any effect of BQCA in vivo is most likely mediated by its interaction with the M_1 mAChR. Nonetheless, to ensure that there is no specific on-target allosteric interaction between BQCA and the selected antipsychotics, we performed competition-binding experiments using the radioligand [^{3}H]NMS on CHO FlpIn cells stably expressing the human M_1 mAChR. As expected, clozapine displayed submicromolar affinity for the M_1 mAChR (K_i = 0.3 μM; Fig. 1A). In contrast, neither haloperidol nor aripiprazole was able to fully inhibit [^{3}H]NMS binding, even at a concentration of 100 μM. Although 10 μM BQCA caused a significant (60-fold) increase in ACh affinity, in agreement with our previous findings (Abdul-Ridha et al., 2014), BQCA had no effect on the affinity of clozapine (Fig. 1B); BQCA also had no effect on haloperidol and aripiprazole (data not shown). Therefore, BQCA does not modulate the binding of the selected antipsychotics at the M_1 mAChR.

Brain Exposure of BQCA. The apparent brain/plasma ratio from our in vivo exposure study (data not shown) was consistent with that observed previously (Ma et al., 2009; Shirey et al., 2009; Gould et al., 2015). Also, there was a clear dose-dependent increase in brain exposure in all cohorts of mice with BQCA administrated via subcutaneous or intraperitoneal injection (Supplemental Fig. 4A). Of note, all C57Bl/6J mice used in the behavioral studies reported in the main text received BQCA via the subcutaneous route. In the last PPI study using $M_1^{+/+}$ and $M_1^{-/-}$ mice (see below), the animals received BQCA dissolved in DMSO-based vehicle via intraperitoneal injection, as in vivo exposure data indicated that this method of administration resulted in higher overall exposure of BQCA in this substrain (Supplemental Fig. 4B). Importantly, as shown in Supplemental Fig. 4C, in no instance was the brain exposure of either BQCA or any of the antipsychotics increased when mice were dosed with both classes of compound. Therefore, any observed behavioral effects of the combination are not a result of increased exposure at their respective sites of action.

BQCA Alone Does Not Rescue MK-801–Induced Disruptions in PPI. The main effect of MK-801 in all cohorts of mice (Table 1) confirmed a significant PPI disruption in MK-801–treated mice (Figs. 2 and 3). However, there was an absence of a BQCA effect in the BQCA-alone cohort, suggesting that BQCA alone did not have any substantial rescuing effects on PPI disruption induced by MK-801 (Fig. 2B).

Combination of Subeffective Doses of BQCA with Typical or Atypical Antipsychotics Produces Significant Reversals in MK-801–Induced Disruptions in PPI. As indicated in the *Materials and Methods*, the dose of haloperidol (0.25 mg/kg) was specifically chosen from prior studies to represent a subeffective dose level in the PPI studies. In the BQCA plus haloperidol cohort, there was a significant BQCA × haloperidol effect (Table 1) as an indication of a synergistic effect between the two compounds. Post hoc analysis (Table 2) showed that MK-801–induced disruption in PPI was not fully reversed by either haloperidol alone or a combination of haloperidol with 1–10 mg/kg BQCA. However, a complete reversal of MK-801–induced PPI disruption was observed when the subeffective dose of haloperidol was combined with 20 mg/kg BQCA (Fig. 3A). Similarly, clozapine (2 mg/kg) alone and in combination with 1–10 mg/kg BQCA failed to fully reverse MK-801–induced PPI disruption. However, a significant reversal was observed with the combination of clozapine and BQCA (20 mg/kg) (Fig. 3B), although there was an absence of BQCA by clozapine interaction in the ANOVA, indicating that both BQCA and clozapine were likely behaving additively rather than synergistically. Comparable trends were also observed in the aripiprazole plus BQCA cohort, but only at the highest prepulse tested (i.e., pp18) (Fig. 3C; Table 2). In this instance, the significant prepulse by BQCA by aripiprazole interaction (Table 1) indicated the presence of a synergistic effect of PPI measured at pp18. Taken together, these results reveal that

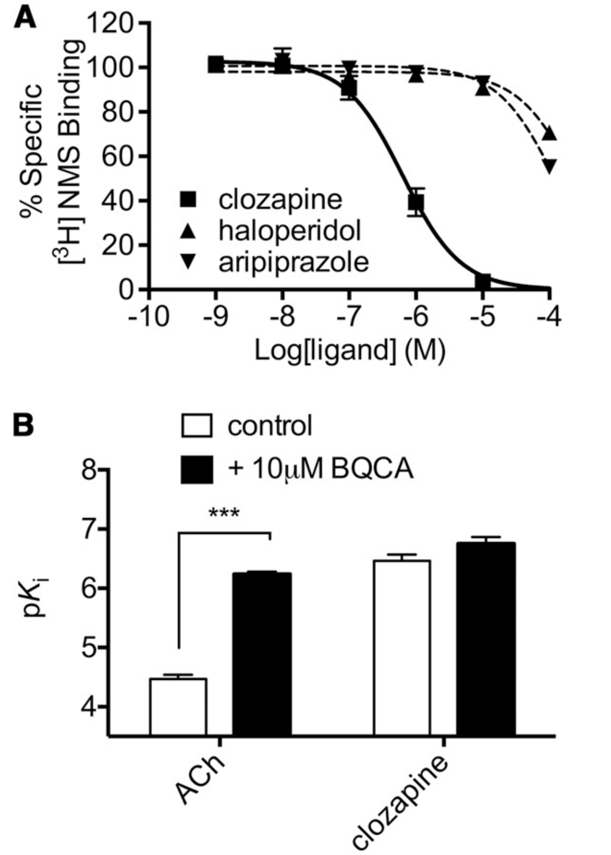

Fig. 1. BQCA does not allosterically modulate antipsychotics at the M_1 mAChR. (A) Antipsychotic affinity was measured at the human M_1 mAChR stably expressed in CHO cells using competition binding experiments with the antagonist [^{3}H]NMS. Clozapine (pK_B = 6.46 ± 0.10) displayed significant affinity for the M_1 mAChR. In contrast, even at a maximal concentration of 100 μM, haloperidol and aripiprazole were unable to completely displace [^{3}H]NMS. (B) Competition binding studies between ACh or clozapine with [^{3}H]NMS were performed in the absence (open bars) and presence (filled bars) of 10 μM BQCA. BQCA (10 μM) caused a significant 60-fold increase in ACh affinity ($P < 0.001$, two-tailed t test) but had no significant effect on the affinity of clozapine.

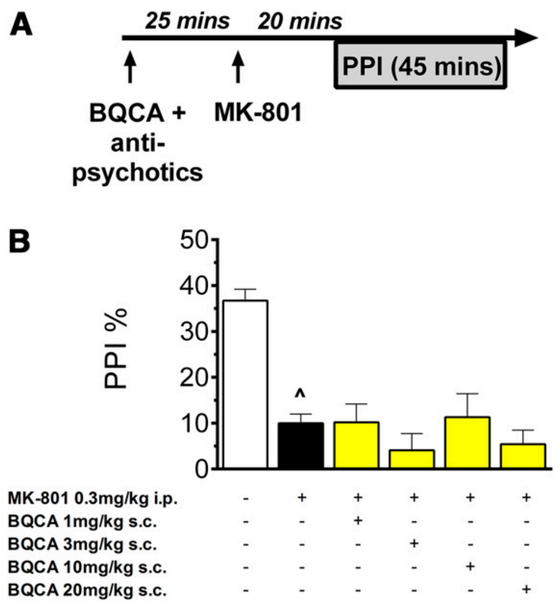

Fig. 2. BQCA alone has minimal effects on MK-801–induced disruptions in PPI. (A) A timeline of drug treatment and PPI testing is presented. MK-801 (0.3 mg/kg, i.p.) was administered 25 minutes after BQCA and antipsychotics, and mice were subjected to PPI testing 20 minutes after MK-801 treatment. (B) MK-801 (0.3 mg/kg, i.p.) induced significant PPI disruption in C57Bl/6J mice. BQCA (0–20 mg/kg, s.c.) did not reverse the MK-801–disrupted PPI. $^{\wedge}P < 0.05$ (for significant MK-801– induced disruption). Data are presented as means ± S.E.M. (vehicle– and MK-801–only controls, n = 15–17, which were reused in each cohort set, in this figure and in Fig. 3; and other treatment groups, n = 9–10).

BQCA enhanced subeffective doses of commonly used antipsychotics to produce a rescue of PPI deficits in MK-801–treated mice.

BQCA Enhanced the Rescuing Effect of Clozapine or Aripiprazole, But Not That of Haloperidol, in the Y-Maze Test. To explore memory performance, the duration spent (in seconds) in novel and familiar arms for each treatment group was analyzed (Table 1). There was a main arm effect and a significant arm × MK-801 interaction, indicating disruption of short-term memory in the Y-maze test by MK-801 (Table 1). Further analysis revealed differences between the novel and familiar arms in vehicle-treated mice (P = 0.002; Fig. 4B). In MK-801–treated mice, pretreatment with 10 mg/kg BQCA and 1 mg/kg clozapine (P = 0.007; Fig. 5B), or 5 mg/kg BQCA and 0.125 mg/kg aripiprazole (P = 0.026; Fig. 5C), also showed novel versus familiar arm differences, which is indicative of normal memory function as seen in vehicle control mice (Fig. 5). There were no differences in the other treatment groups, including BQCA alone or in combination with haloperidol (Fig. 5A). These results demonstrate a synergistic effect between BQCA and the atypical antipsychotics, clozapine or aripiprazole, on memory function, which was not seen with BQCA on its own or in combination with haloperidol, in this acute memory disruption model.

M_1 mAChR Is Required for the Combined Effect of BQCA and Clozapine in Reversing PPI Deficits. To confirm the requirement of the M_1 mAChR for the observed combined effect, we repeated selected key BQCA and clozapine interaction studies in mice lacking the M_1 mAChR; this also necessitated testing in different background mouse strains. The combined effect between BQCA and clozapine seen in reversing MK-801–induced PPI deficits was reproducible in C57Bl/6J mice using this administration method (Supplemental Fig. 5). Moreover, consistent with previous findings (Matsuo et al., 2010), we noted that both $M_1^{+/+}$ and $M_1^{-/-}$ mice on a C57Bl/6N background exhibited a higher PPI compared with the C57Bl/6J mice used in the preceding studies. PPI disruption was induced to a similar extent by 0.3 mg/kg MK-801 in both $M_1^{+/+}$ and $M_1^{-/-}$ mice. A partial,

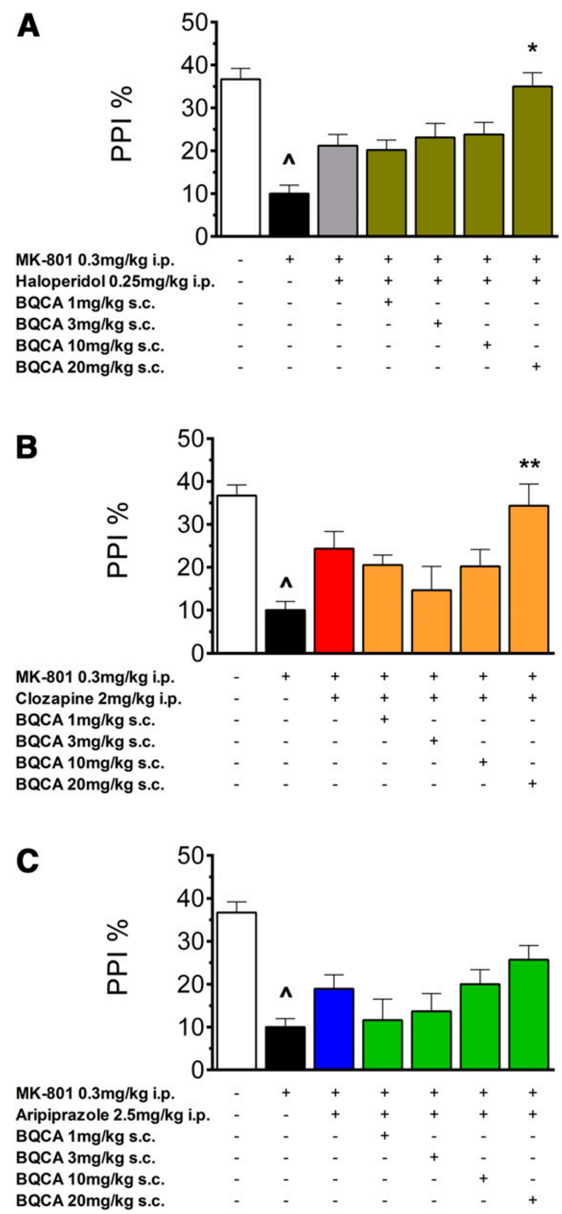

Fig. 3. Combination of subeffective doses of BQCA and haloperidol or clozapine can reverse MK-801–induced disruption in average PPI. (A and B) Both haloperidol (0.25 mg/kg) and clozapine (2 mg/kg) did not reverse MK-801–induced PPI disruption; however, the combination with BQCA (20 mg/kg, s.c.) resulted in significant reversal of PPI disruption in C57Bl/6J mice. (C) The combination of BQCA (20 mg/kg, s.c.) and aripiprazole did not produce a reversal in average PPI, except PPI measured at pp18 (Table 2). $^{*}P < 0.05$ (for difference with MK-801 post hoc comparisons); $^{**}P < 0.001$ (for difference with MK-801 post hoc comparisons); $^{\wedge}P < 0.05$ (for significant MK-801–induced disruption). Data are presented as means ± S.E.M. (vehicle– and MK-801–only controls, n = 15–17, which were reused in each cohort set, in this figure and in Fig. 2; and other treatment groups, n = 9–10).

but statistically nonsignificant, reversal of the PPI deficit was observed after dosing clozapine alone, consistent with the observation in C57Bl/6J mice (Fig. 6). A qualitatively similar partial reversal was observed after $M_1^{+/+}$ mice received BQCA alone at 20 mg/kg, a trend that was not observed in C57Bl6J mice. This reversal failed to achieve statistical significance and was not observed in subsequent studies with further cohorts of $M_1^{+/+}$ mice. The most important finding, however, was that MK-801–disrupted PPI was rescued by the combination of BQCA (20 mg/kg) and clozapine (3 mg/kg) in the $M_1^{+/+}$ mice, and there was a complete absence of such rescue in the $M1^{-/-}$ mice receiving the same treatment (Fig. 6; Table 3). These results demonstrate the selectivity of BQCA in vivo and also confirm that the M_1 mAChR is required in order to observe efficacy enhancement of clozapine actions.

Discussion

This study provides proof of concept that the combination of an M_1 mAChR PAM with different antipsychotic drugs can yield synergistic (or additive) efficacy in two animal behavioral models that are considered to reflect aspects of schizophrenia. These effects are not mediated by on-target allosteric interactions at the M_1 mAChR between BQCA and the antipsychotics, but activation of the receptor is necessary because the interaction between BQCA and clozapine in reversing MK-801–induced PPI disruption is lost in $M_1^{-/-}$ mice. If these findings can be generalized to other aspects or models of schizophrenia, then this may provide impetus for future consideration of the use of M_1 mAChR PAMs as potential add-on therapies to selected existing drug regimens.

Haloperidol is a first-generation antipsychotic agent, representative of the requirement for potent dopamine D_2 receptor

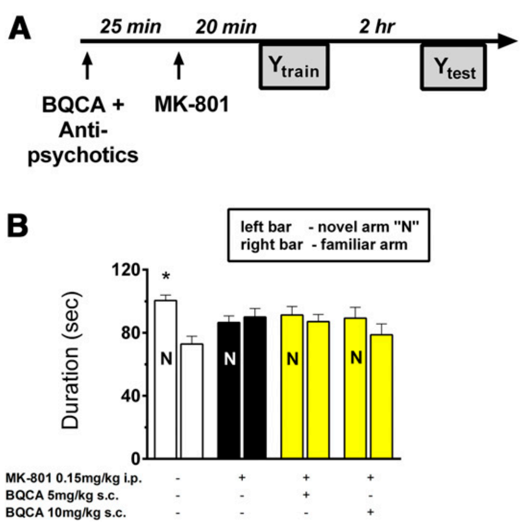

Fig. 4. MK-801 induces spatial memory deficits in C57Bl/6J mice in the Y-maze test. (A) A timeline of drug treatment and Y-maze testing is presented. BQCA and antipsychotics were given 25 minutes prior to MK-801 (0.15 mg/kg) administration, and the mice were then subjected to a Y-maze training session 20 minutes after MK-801 treatment. The retention period was 2 hours (between training [Y_{train}] and testing [Y_{test}] sessions). The duration spent (in seconds) in the novel and familiar arms was used as the measure of memory function. (B) Analysis showed no rescue of memory deficits induced by MK-801 (0.15 mg/kg) for mice pretreated with BQCA alone. *$P < 0.05$ (for difference between the novel and familiar arms as an indication of normal memory function). Data are presented as means ± S.E.M. (n = 10–13 mice per group). N, novel.

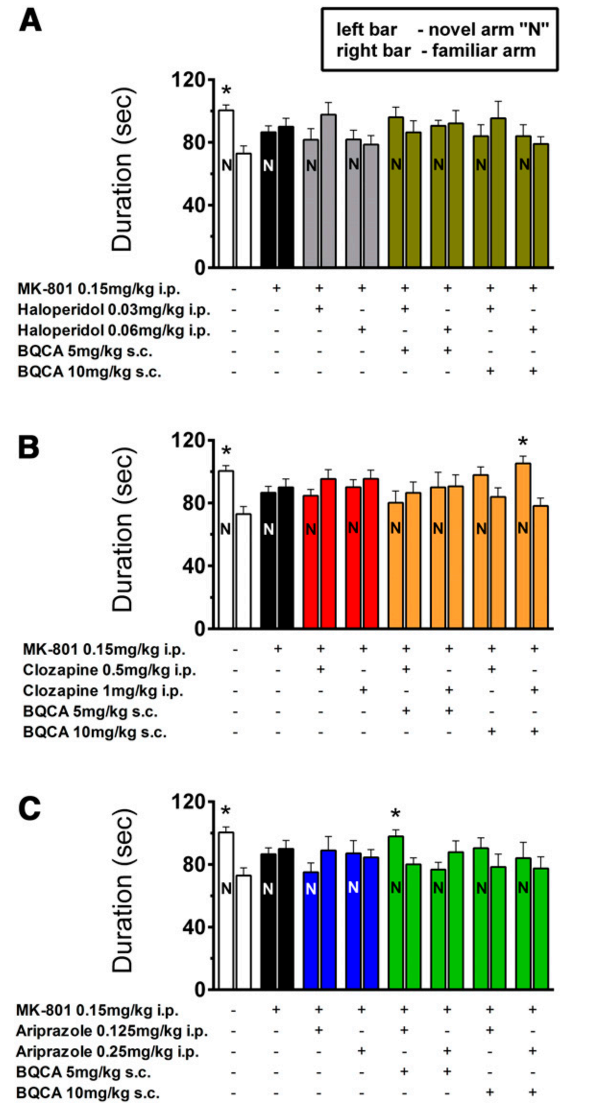

Fig. 5. The combination of BQCA with clozapine or aripiprazole, but not with haloperidol, improves MK-801–induced spatial memory in C57Bl/6J mice in the Y-maze test. (A) Mice pretreated with BQCA combined with haloperidol showed no rescue of MK-801–induced memory disruption. (B and C) In contrast, mice treated with combinations of BQCA (10 mg/kg, s.c.) and clozapine (1 mg/kg), or BQCA (5 mg/kg, s.c.) and aripiprazole (0.125 mg/kg), showed rescue of the MK-801 (0.15 mg/kg) memory disruptive effect. *$P < 0.05$ (for difference between the novel and familiar arms as an indication of normal memory function). Data are presented as means ± S.E.M. (n = 10–13 mice per group). N, novel.

antagonism in treating positive schizophrenic symptoms (Mansbach et al., 1988; Conn et al., 2008; Miyamoto et al., 2012). Clozapine was chosen as an atypical second-generation antipsychotic with a broad polypharmacology profile (Miyamoto et al., 2005; Nasrallah, 2008), whereas aripiprazole was selected as a third-generation agent that acts on D_2 receptors as a partial agonist with a lower propensity toward adverse metabolic side effects (Nasrallah, 2008; Cui et al., 2010). None of these drugs, however, are effective at treating cognitive deficits associated with schizophrenia (Lieberman et al., 2005; Lublin et al., 2005). However, therapeutic targeting has not been limited to D_2 receptors (Conn et al., 2008; Owen et al., 2016). For example, agents targeting mAChRs that are highly expressed in affected

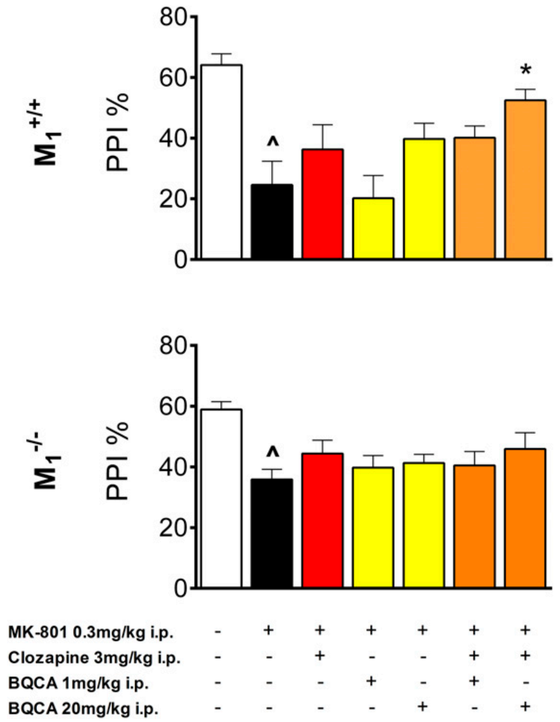

Fig. 6. The enhancement of efficacy of clozapine by BQCA in reversing PPI deficits is absent in M_1 mAChR knockout mice. MK-801 (0.3 mg/kg) induced PPI disruption in both $M_1^{+/+}$ mice (top) and $M_1^{-/-}$ mice (bottom). Despite differences in the PPI profile between C57Bl/6J and $M_1^{+/+}$ (C57Bl/6N background) mice, an additive effect of BQCA (20 mg/kg, i.p.) and clozapine (3 mg/kg) was still observed in the $M_1^{+/+}$ mice but not in the $M_1^{-/-}$ mice. The synergetic effect was particularly significant at pp6 (Table 3). $*P < 0.05$ (for difference with MK-801 post hoc comparisons); $\hat{}P < 0.05$ (for significant MK-801–induced disruption). Data are presented as means ± S.E.M. ($M_1^{+/+}$ vehicle and MK-801 controls, n = 15–16; $M_1^{+/+}$ treatment groups, n = 8–9; $M_1^{-/-}$ vehicle and MK-801 controls, n = 13–15; and $M_1^{-/-}$ treatment groups, n = 8).

regions, such as the prefrontal cortex and hippocampus, have received attention for their potential in rescuing cognitive dysfunction (Bymaster et al., 2002; Shekhar et al., 2008; Bridges et al., 2010; Barak and Weiner, 2011; Bolbecker and Shekhar, 2012). In this study, we specifically focused on NMDAR antagonism by MK-801 as a means of modeling sensorimotor deficits and short-term memory disruption due to glutamatergic hypofunction (Gilmour et al., 2012), as opposed to pharmacological interventions such as amphetamine that model positive symptom domains but are more limited for studying negative or cognitive domains (Young et al., 2010; Katsnelson, 2014). For behavioral models, the PPI test measures sensorimotor gating in the brain, as it is impaired in patients with schizophrenia, and has been used in animals to identify and understand antipsychotic efficacies of clinically available drugs (Keith et al., 1991; Bakshi et al., 1994; Swerdlow et al., 1994; Swerdlow and Geyer, 1998; Cadenhead et al., 1999; Young et al., 2010; Moore et al., 2013). The Y-maze test is a short-term spatial memory paradigm for assessing hippocampal-dependent memory function (Conrad et al., 1996, 1997; Choy et al., 2008; Shipton et al., 2014), which is highly vulnerable to pathologic factors such as stress (Heckers et al., 1998; Harrison, 2004; Sweatt, 2004; van Erp et al., 2004, 2016; Tanskanen et al., 2005; Vakalopoulos, 2006; Velakoulis et al., 2006; Choy et al., 2008) and is impaired from the earliest stages of schizophrenia (Wood et al., 2002, 2003). Therefore, the Y-maze test has potential to provide preclinical insights into the effects of combining BQCA and antipsychotics on brain regions affected along the pathologic trajectory of schizophrenia (Heckers et al., 1998; Weinberger, 1999; Lipska and Weinberger, 2002; Harrison, 2004; Sweatt, 2004; van Erp et al., 2004; Harrison and Weinberger, 2005; Tanskanen et al., 2005; Velakoulis et al., 2006)

In general, BQCA alone had minimal effects in both behavioral models. With the exception of the highest dose of clozapine (5 mg/kg), which also affected the animal startle response, the doses of haloperidol, clozapine, and aripiprazole used in this study were insufficient to restore normal PPI after MK-801 treatment, consistent with findings from previous studies (Bakshi et al., 1994; Bast et al., 2000; Ishii et al., 2010). However, it should be noted that this subeffective dosing was necessary to reveal the effect of combination with BQCA.

TABLE 3

PPI measured with prepulses at 6, 12, and 18 db above the background in $M_1^{+/+}$ and $M_1^{-/-}$ (C57Bl/6 Ntac background) mice, which received BQCA via the intraperitoneal route

Data are presented as means ± S.E.M.

Group	PPI		
	pp6	pp12	pp18
		%	
$M_1^{+/+}$ mice			
Vehicle control	46.8 ± 3.9	69.0 ± 4.1	76.4 ± 3.6
MK-801	0.9 ± 8.6*	27.8 ± 9.3*	45.1 ± 7.1*
+ 1 mg/kg BQCA	−2.4 ± 6.1	28.1 ± 8.4	35.0 ± 10.7
+ 20 mg/kg BQCA	14.0 ± 6.3	42.2 ± 5.3	63.0 ± 4.9
MK-801 + 3 mg/kg clozapine	10.9 ± 9.6	39.8 ± 9.4	58.3 ± 6.1
+ 1 mg/kg BQCA	19.4 ± 4.5	44.4 ± 4.6	59.5 ± 5.2
+ 20 mg/kg BQCA	34.7 ± 5.0**	54.0 ± 4.8	68.8 ± 2.7
$M_1^{-/-}$ mice			
Vehicle control	35.4 ± 4.0	65.7 ± 2.4	75.5 ± 2.4
MK-801	8.7 ± 4.8*	41.9 ± 4.8*	57.0 ± 2.6*
+ 1 mg/kg BQCA	14.3 ± 5.9	45.2 ± 4.5	59.9 ± 4.6
+ 20 mg/kg BQCA	17.4 ± 4.2	47.3 ± 4.6	59.1 ± 3.2
MK-801 + 3 mg/kg clozapine	16.4 ± 6.0	50.9 ± 5.6	65.8 ± 3.4
+ 1 mg/kg BQCA	16.7 ± 5.7	46.7 ± 4.9	58.0 ± 4.8
+ 20 mg/kg BQCA	23.4 ± 8.3	51.5 ± 4.7	62.7 ± 6.2

$*P < 0.05$ (versus vehicle control − MK-801 effect); $**P < 0.05$ (versus MK-801 control at the same prepulse).

Lowering doses of antipsychotics also brings potential benefits, because the side-effect burden would be lessened if therapeutically effective lower doses were to be administered clinically. The fact that coadministration of the M_1 mAChR PAM with each of the three classes of antipsychotic rescued the disrupted PPI response indicates that sensorimotor gating deficits can be particularly amenable to combination therapy, even in the case of clozapine, which itself has affinity for the M_1 mAChR. Presumably, potentiation of endogenous ACh affinity at this receptor by BQCA, however, would reduce M_1 mAChR occupancy by clozapine (and its metabolites) in preference for the full agonist, ACh. Importantly, we confirmed that the combined effect of BQCA and clozapine was reproducible in the $M_1{}^{+/+}$ mice but lost in the $M_1{}^{-/-}$ mice, despite differences in the overall PPI profile between the C57Bl/6 substrains (Matsuo et al., 2010).

In addition to sensorimotor gating deficits, spatial memory dysfunction, which is mostly associated with decline of hippocampal function, has been commonly observed in patients with schizophrenia, even at early onset stages (Saykin et al., 1994; Weinberger and Gallhofer, 1997; Heckers et al., 1998; Wood et al., 2002; Bertolino et al., 2003; Egeland et al., 2003; Brewer et al., 2005; Bartholomeusz et al., 2011). In contrast with the PPI studies, the atypical antipsychotics, clozapine and aripiprazole, but not haloperidol, showed an enhancement of efficacy by BQCA in reversing MK-801–induced spatial memory disruption in the Y-maze. The hippocampus has a high expression of M_1 mAChRs, which are important for memory formation in rodents (Talts et al., 1998; Anagnostaras et al., 2003; Lee and Kesner, 2003; Newell et al., 2007; Mauck et al., 2010; Peng et al., 2012). Indeed, BQCA enhances baseline spatial and object recognition memory in rodents (Chambon et al., 2011, 2012), reverses scopolamine-disrupted fear and working memory, and restores memory function in Tg2576 mice (Ma et al., 2009; Shirey et al., 2009; Chambon et al., 2012). To our knowledge, however, ours is the first study to extend these effects to rescue of MK-801–induced memory impairments, albeit in the presence of a coadministered antipsychotic. Interestingly, another recent study showed that a PAM of the M_4 mAChR subtype could rescue MK-801–induced impairments in a touchscreen-based memory retrieval task (Bubser et al., 2014), and earlier work established that the cholinesterase inhibitor, donepezil, can also rescue MK-801–disrupted fear memory (Csernansky et al., 2005).

In terms of the brain regions mediating the interactions between the M_1 mAChR PAM and antipsychotics in our models, it is likely that cortical and striatal regions are involved in the PPI effects, since they express the M_1 mAChR (Han et al., 2008; Ma et al., 2009; Peng et al., 2012) and have been implicated in regulation of sensorimotor gating mechanisms (Swerdlow et al., 1990, 2001; Geyer et al., 2001; Braff, 2010). Although the M_1 mAChR is also found in the hippocampus, it is unlikely to be as important in the observed effects, as MK-801–induced PPI disruption does not involve this region and, if anything, cholinergic activation in the hippocampus can itself lead to some PPI disruption (Caine et al., 1991, 1992; Zhang et al., 2000). In contrast, the Y-maze test is substantially hippocampal dependent (Conrad et al., 1996, 2007; Choy et al., 2010; Shipton et al., 2014). Nonetheless, the possible involvement of cortical regions in both behaviors should not be overlooked, as cortical, striatal, and hippocampal networks play an important role in higher cognitive function (Turnock and Becker, 2008; Pennartz et al., 2011; Britt et al., 2012).

An area of ongoing discussion is the issue of whether "single-target bullets" or "multifunction drugs" will lead to more effective medications for schizophrenia (Pantelis and Barnes, 1996; Roth et al., 2004; Miyamoto et al., 2012). What remains indisputable, however, is that schizophrenia is a disorder that requires drugs capable of targeting multiple symptom domains, in particular cognitive deficits. Moreover, the incidence of adverse effects remains a major problem for patients undergoing chronic treatment with existing antipsychotics (Nasrallah, 2008). Both of these issues may conceivably be addressed by judicious combination therapies. For instance, although clozapine was used as proof of concept in our study, it may not be amenable for combination therapies in practice due to its known on-target mAChR effects, whereas other atypical agents (e.g., aripiprazole or risperidone, which have minimal mAChR activity; Roth et al., 2004) may represent more appropriate candidates. Furthermore, the addition of a highly selective mAChR PAM (i.e., single-target bullet) may yield an increase in symptom domain coverage together with an existing antipsychotic (i.e., multifunction drug). Although these issues remain speculative at this time, our study shows that we can observe synergistic or additive efficacy in our models while essentially retaining normal exploratory behaviors in the Y-maze test and normal body reflex upon acoustic startle stimulation in the PPI test. Thus, in addition to offering novel approaches to targeting G protein–coupled receptors in a highly selective manner to address unmet medical needs, allosteric modulators may potentially allow extension of the clinical efficacy of existing therapeutics and warrant further study in this regard.

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Authorship Contributions

Participated in research design: Choy, Shackleford, Malone, Lane, Christopoulos.

Conducted experiments: Choy, Shackleford, Mistry, Patil, Scammells, Lane.

Performed data analysis: Choy, Shackleford, Malone, Lane.

Wrote or contributed to the writing of the manuscript: Choy, Shackleford, Malone, Mistry, Scammells, Langmead, Pantelis, Sexton, Lane, Christopoulos.

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SUPPLEMENTAL DATA

Title: Positive allosteric modulation of the muscarinic M_1 receptor improves efficacy of antipsychotics in mouse glutamatergic deficit models of behavior

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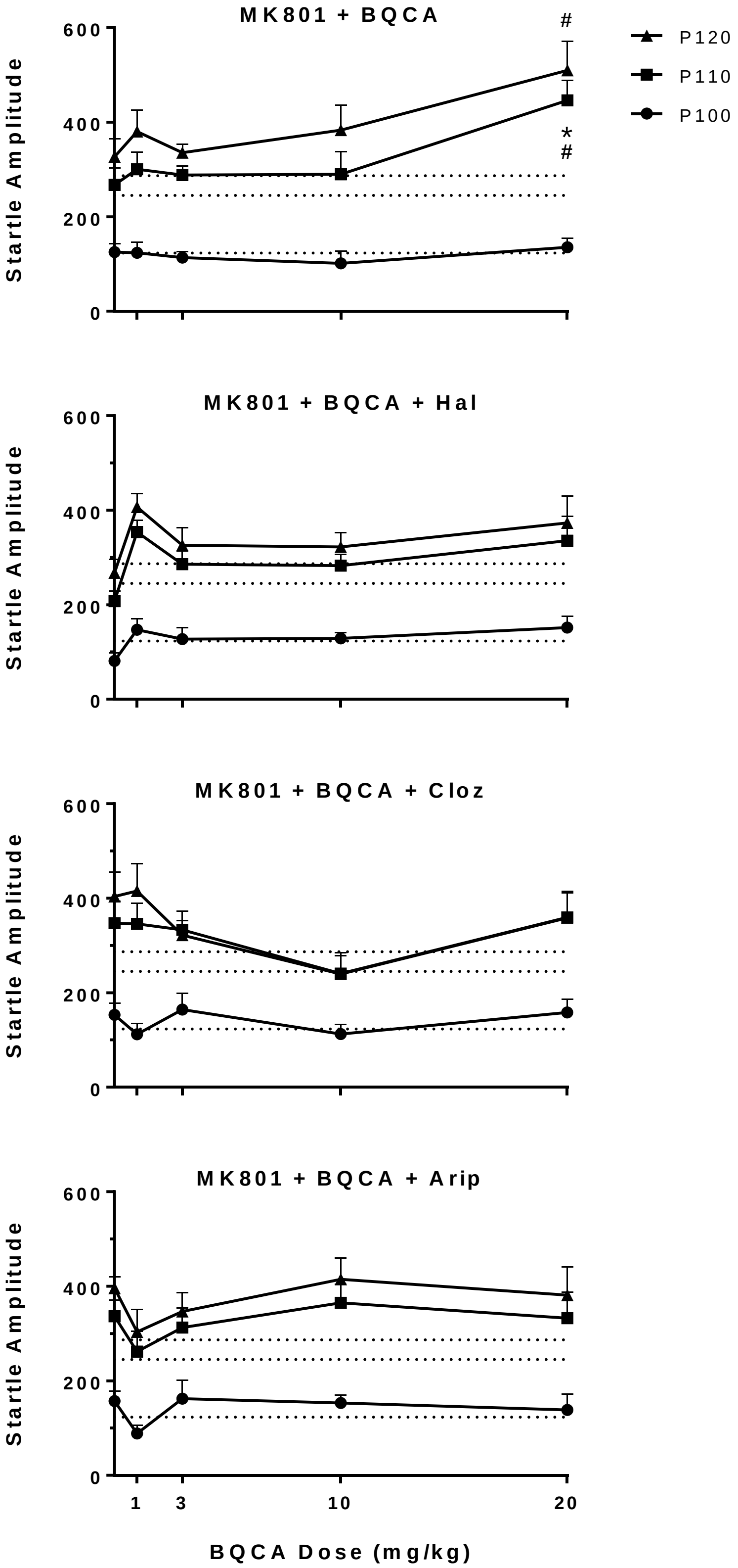
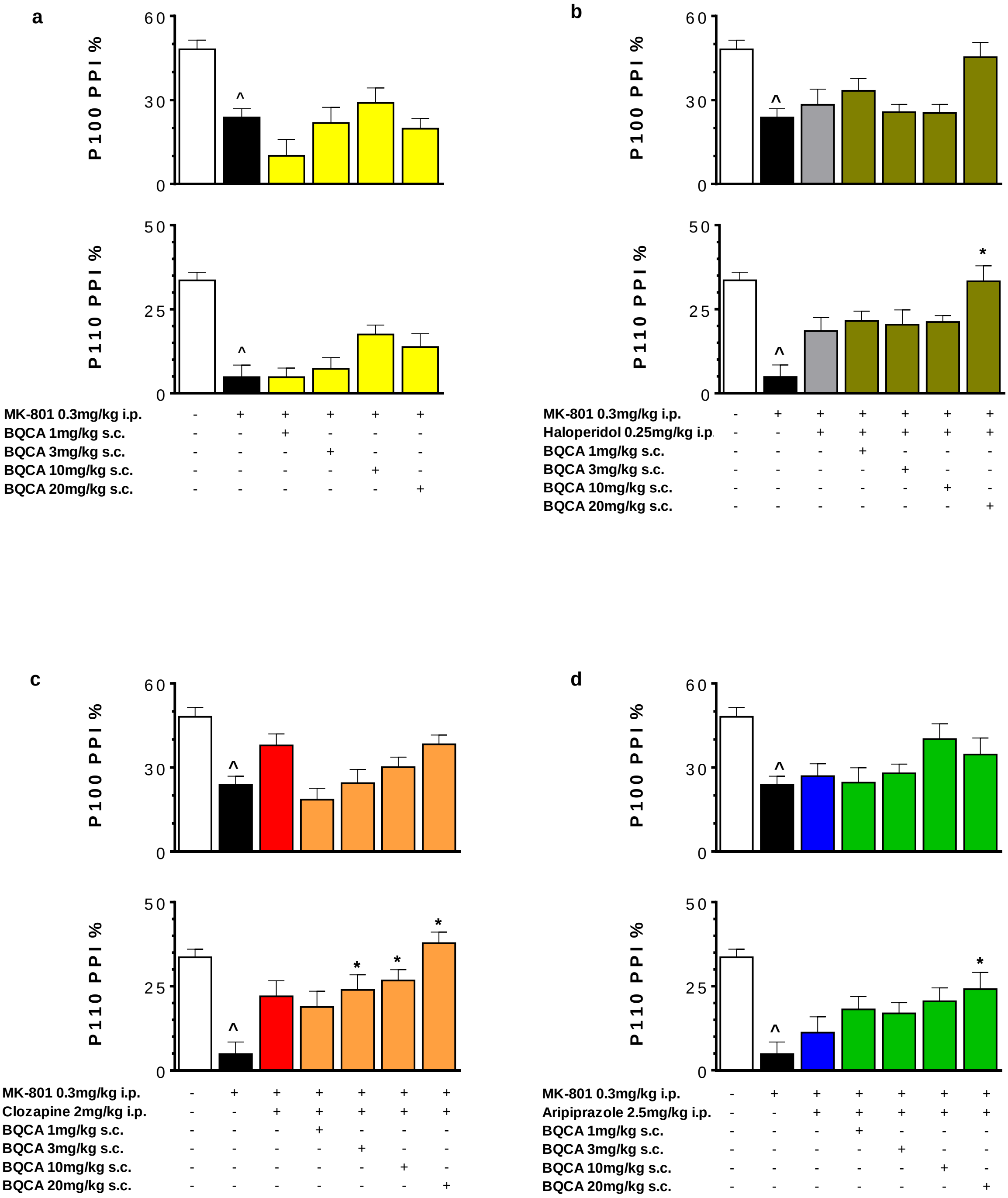

MK801 + BQCA
MK801 + BQCA + Hal
MK801 + BQCA + Cloz
MK801 + BQCA + Arip
Startle Amplitude
0
200
400
600
#
*
P120
P110
P100
1
3
10
20
BQCA Dose (mg/kg)

Supplemental Fig.1

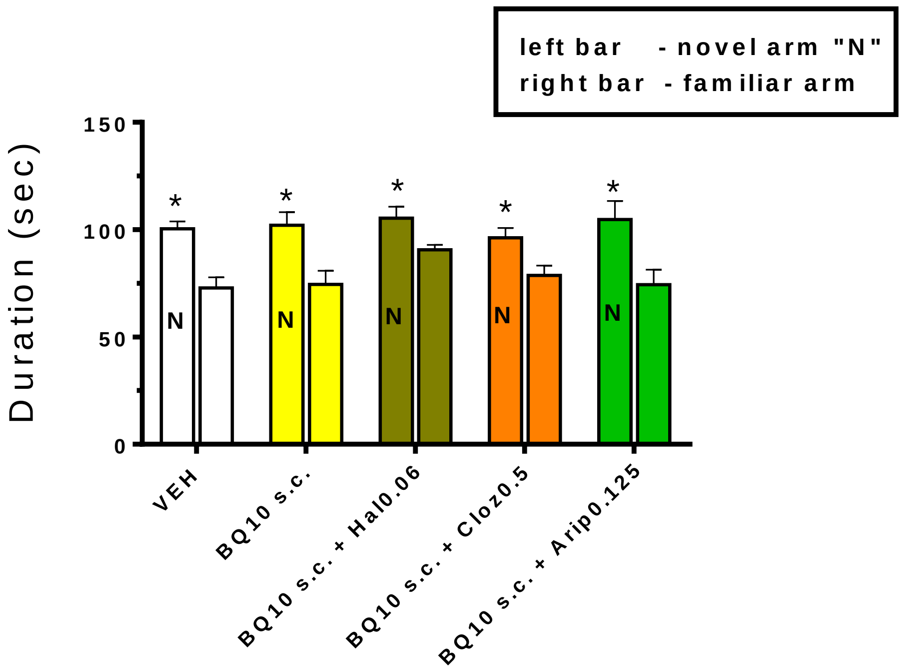
Startle amplitude measured at pulse alone (p100, 110 and 120) during the PPI test remained unaffected by drug pretreatment, except mice that were treated by 20 mg/kg (s.c.) of BQCA and MK-801 (0.3 mg/kg). In particular, the higher startle amplitude is observed in tested mice measured at 110 and 120db stimulus (top panel).
Data are mean ± SEM, * $p < 0.05$ vs MK-801 control, and $^{\#}$ $p < 0.05$ vs vehicle control (-----).



Supplemental Fig. 2.

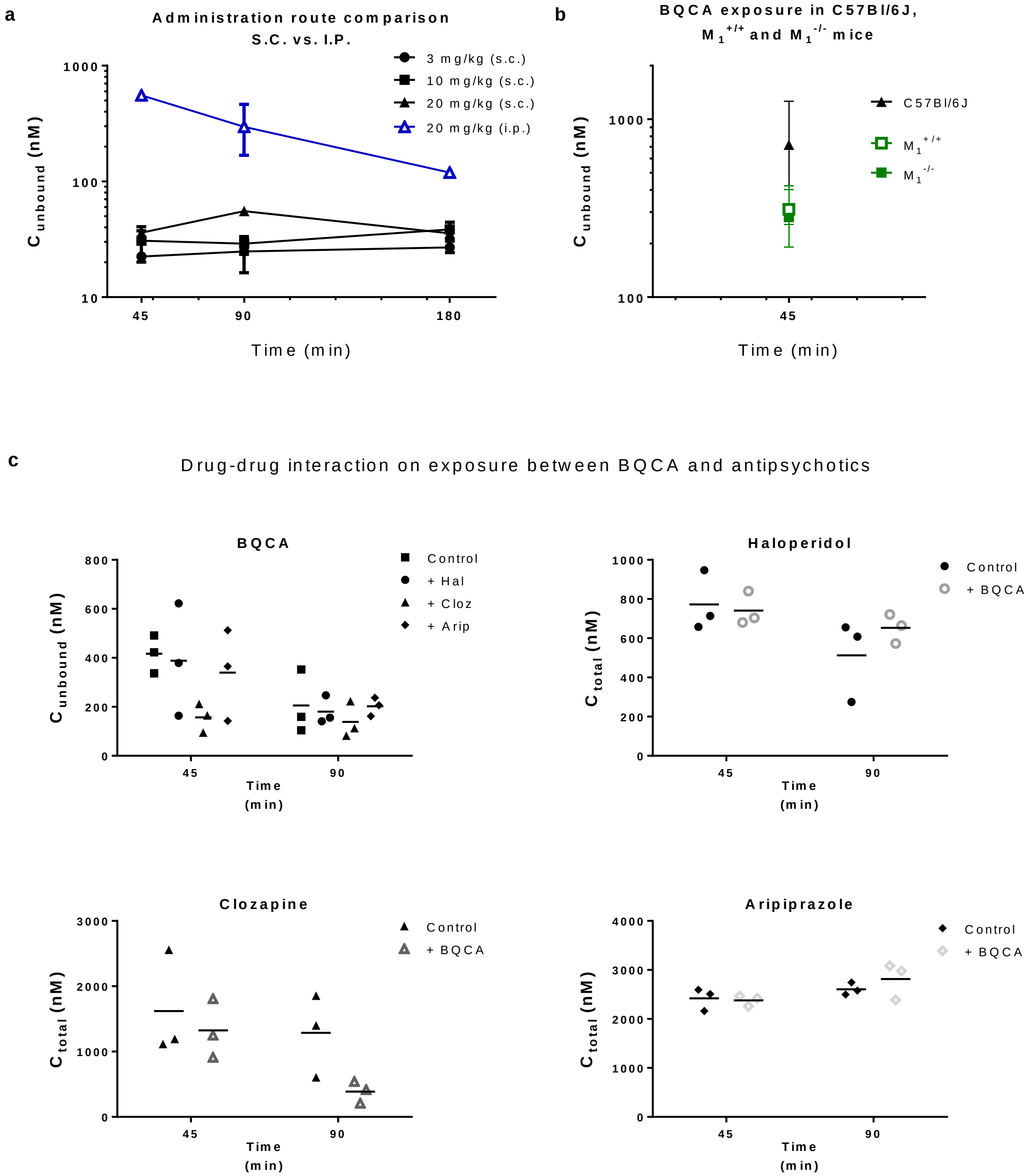
PPI measured at p100 and p110 exhibited a similar effect as observed at p120. As mentioned in Materials and Methods, mice were tested in PPI of startle elicited by acoustic stimulus of 100, 110 and 120db, and prepulses. PPI measured at 120db is presented in the main text. PPI measured at p100 and p110 either shared a similar reversal seen in p120 or a trend of reversal, by combination of BQCA and antipsychotics.

Data are mean ± SEM, ^ $p < 0.05$ for difference between vehicle vs MK-801 controls, as indication of PPI disruption by MK-801; * $p < 0.05$ for difference between groups vs. MK-801 control for a significant reversal of PPI disruption.



Supplemental Fig. 3.

Memory function was not affected by pretreatment of BQCA alone, or combined BQCA and antipsychotics treatment, in mice without MK-801 treatment, whereas C57Bl/6J mice received BQCA (10 mg/kg s.c.) and clozapine (1 mg/kg), or aripiprazole (0.25 mg/kg) exhibited insufficient exploratory activity, and therefore excluded.
* $p < 0.05$ for difference between novel and familiar arm as an indication of normal memory function.

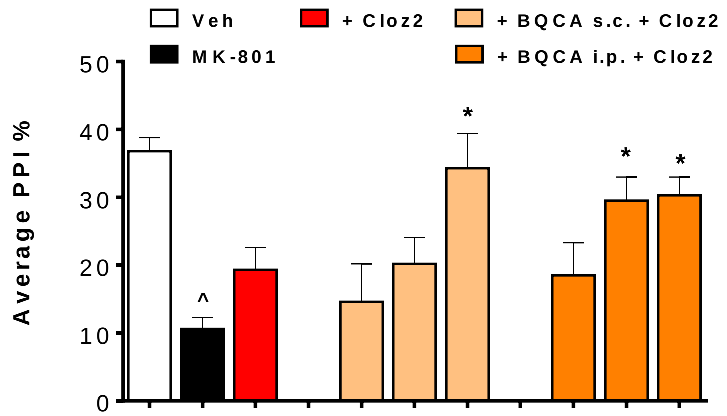


Supplemental Fig 4.

Assessment of BQCA exposure in mouse brain in the absence or presence of antipsychotics. (a) *Experiment 1*: Unbound concentrations ($C_{unbound}$ in nM) of BQCA in brains of C57Bl/6J mice after s.c. (Suspensions in 50% Pharmasolve® based vehicle) or i.p.

(Solutions in 15% DMSO based vehicle) injection. I.p. injection of BQCA resulted in a higher drug delivery to the brain than s.c. injection. (b) *Experiment 2*: $C_{unbound}$ of BQCA in $M_1^{+/+}$ and $M_1^{-/-}$ mice (C57Bl/6N background) compared to C57Bl/6J mice, suggests that BQCA brain exposure was lower in the C57Bl/6N mice than C57Bl/6J mice, and that there was no difference between the genotypes ($M_1^{+/+}$ vs. $M_1^{-/-}$). (c) *Experiment 3*: $C_{unbound}$ of BQCA in brain was assessed in C57Bl/6J mice after s.c. injection of BQCA (20 mg/kg), with or without coadministration of haloperidol (0.25 mg/kg i.p.), clozapine (2 mg/kg i.p.) or aripiprazole (top left graph). C_{total} of the antipsychotics (haloperidol, clozapine or aripiprazole) in brain was also assessed with or without coadministration of BQCA (20 mg/kg s.c.) (top right and bottom graphs). There was no enhancement of brain exposure of BQCA or antipsychotics when mice were dosed with both compounds.

$C_{unbound}$ was calculated as C_{total} * f_u using published f_u values of 0.126 in brain, respectively (Gould et al., 2015). Each value represents the mean within the range of data of *n = 2-3* animals per group.



Route of administration				s.c.	s.c.	s.c.	i.p.	i.p.	i.p.
Injection dose (mg/kg)				3	10	20	0.1	1	20
nM @ 45min				22.5	30.7	36.0	na	39.1	477.4

Supplemental Fig. 5.

Reversal of disrupted PPI induced by MK-801 in C57Bl/6J mice receiving BQCA via i.p. administration using a lower dosing range, as BQCA administrated via .i.p. route (DMSO containing vehicle) allowed a higher brain exposure compared to s.c. route. There was a similar $C_{unbound}$ (in nM) of BQCA in the brain observed between BQCA administrated via i.p. and s.c. (39.1 ± 4.6 nM and 36.0 ± 4.5 nM respectively). Furthermore, a high dose of BQCA via i.p. (20 mg/kg) also produced a similar reversal effect on MK-801 treatment.

Methods and data analysis: A separated cohort of C57Bl/6J received BQCA via i.p. route, together with vehicle and MK-801 alone controls. For data analysis, this PPI data was combined with data obtained from BQCA (s.c.) + clozapine cohort (Fig. 3b). The PPI% of both vehicle groups were approximately the same: BQCA s.c. cohort = 36.7 ± 2.5% and BQCA i.p. cohort = 36.9 ± 3.2%. ANOVA test were performed on the combined PPI data. BQCA ($p < 0.001$), clozapine ($p = 0.022$) and MK-801 ($p < 0.001$) effects were found, and then followed with Tukey's post hoc comparisons, where ^ $p < 0.05$ for significant PPI disruptive effect of MK-801, and * $p < 0.05$ for difference with MK-801.