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Cognitive Improvements in a Mouse Model with Substituted 1,2,3-Triazole Agonists for Nicotinic Acetylcholine Receptors

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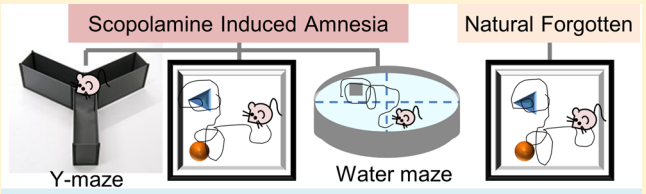
Supporting Information

ABSTRACT: The α7 nicotinic acetylcholine receptor (nAChR) is a recognized drug target for dementias of aging and certain developmental disorders. Two selective and potent α7-nAChR agonists, winnowed from a list of 43 compounds characterized in a companion article (DOI: 10.1021/acschemneuro.5b00058), 5-((quinuclid-3-yl)-1*H*-1,2,3-triazol-4-yl)-1*H*-indole (IND8) and 3-(4-hydroxyphenyl-1,2,3-triazol-1-yl) quinuclidine (QND8), were evaluated for cognitive improvement in both short- and long-term memory. Tacrine, a centrally active acetylcholinesterase inhibitor, and PNU-282987, a congeneric α7 nAChR agonist, were employed as reference standards. Three behavioral tests, modified Y-maze, object recognition test (ORT), and water maze, were performed in scopolamine-induced amnesic mice. Intraperitoneal injection of these two compounds significantly improved the cognitive impairment in a modified Y-maze test (5 μmol/kg for IND8 and 10 μmol/kg for QND8), ORT (10 μmol/kg), and water maze test (25 μmol/kg). For delay induced memory deficit or natural memory loss in mice, IND8 and QND8 at 10 μmol/kg were able to enhance memory comparable to PNU-282987 when evaluated using ORT time delay model. Cognitive enhancement of IND8 and QND8 was mediated through α7-nAChRs as evidenced by its complete abolition after pretreatment with a selective α7-nAChR antagonist, methyllycaconitine. These data demonstrate that IND8 and QND8 and their congeners are potential candidates for treatment of cognitive disorders, and the substituted triazole series formed by cycloaddition of alkynes and azides warrant further preclinical optimization.



Cpd	Minimum Effective Dose (μmol/kg)			
	Y-maze	ORT	Water maze	ORT
IND8	5	10	25	10
QND8	10	10	25	10
PNU-282987	33	-	-	10

KEYWORDS: *α7-nAChR agonist, behavioral memory loss, cognitive improvement, modified Y-maze, object recognition test (ORT), water maze*

Alzheimer's disease (AD) is the most common neurodegenerative disorder in the elderly population; patient deficits encompass cognitive impairments that affect short-term memory, working memory, attention, and also long-term memory, leading to loss of ability to accomplish functions of daily life.[1,2] The hallmarks of this disease are amyloid plaques, neurofibrillary tangles, and neuronal loss.[2] A predominant set of vulnerable neurons in AD are cholinergic neurons.[3] Dysfunction of cholinergic neurons and reduction of nicotinic acetylcholine receptor (nAChR) expression in brain of AD patients support a role for cholinergic therapy in AD.[4,5]

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channel (LGIC) receptors in Cys-loop superfamily. Structurally, these receptors can be divided into 3 domains: an extracellular binding domain, transmembrane region, and intracellular domain. There are multiple subtypes composed of up to 12 different neuronal subunits (α2-α10 and β2-β4) characterized in mammalian and avian systems, which can be divided into homo- and heteromeric pentamers depending on permutations of subunit assembly.[6,7] Differences in subunit combinations confer various functions and regional localizations in the CNS.[6,8] The most abundant nAChRs in cerebral cortex and hippocampus, brain areas associated with cognition and memory, are the α4β2- and α7-subtypes.[9]

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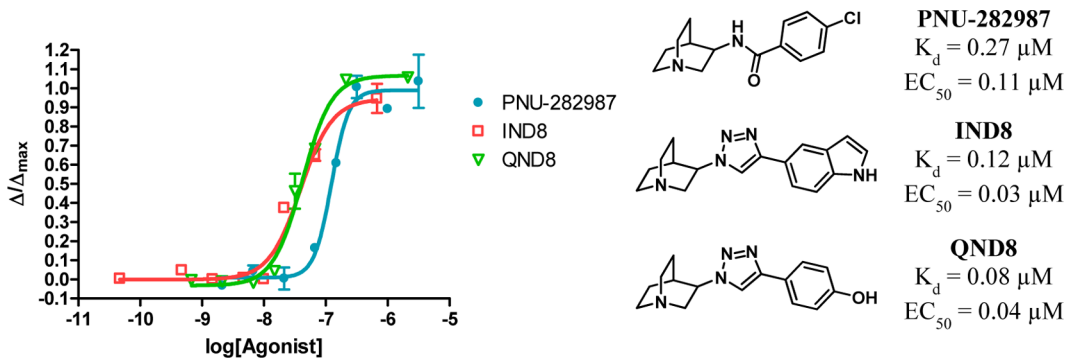

Figure 1. Dose−response curves and structures of evaluated α7-nAChR agonists with dissociation constants (K_d) and agonist potency (EC_{50}) to α7-nAChRs.[23]

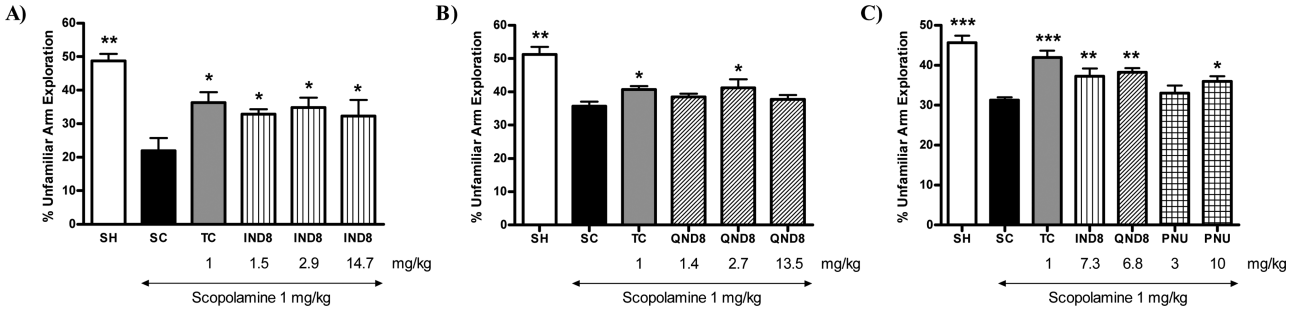

Figure 2. Cognitive improvement in scopolamine-induced amnesia (modified Y-maze). Percentages of unfamiliar arm exploration in modified Y-maze are shown for IND8 (A), QND8 (B), and IND8, QND8, PNU-282987 (C). IND8, QND8, PNU-282987, vehicle (15% Tween 80, SH) and tacrine (TC) were injected 1 h and scopolamine (SC) 30 min before sample phase, respectively. Each mouse was placed at the end of one arm and allowed to access two arms of the Y-maze for 5 min in sample phase. After a 30 min period, the mouse was allowed to explore three arms for 5 min. The number of entries for each arm was recorded and the percentage of unfamiliar arm exploration was calculated. Data are represented as means ± standard errors (SE); n = 8−10 mice/group for IND8 (A), n = 9−10 mice/group for QND8 (B), n = 7−10 mice/group for IND8, QND8, and PNU-282987 (C). *p < 0.05, **p < 0.01, ***p < 0.001 vs scopolamine-treated group (SC), one-way ANOVA with Fisher's LSD post hoc comparison.

The α7-nAChR is a homopentameric receptor with all five subunits surrounding an ion pore that regulates cation permeability, especially to Ca^{2+}.[6] Expression of this receptor subtype is not limited to cerebral cortex and hippocampus, but also found in subcortical limbic regions, thalamic regions, and basal ganglia.[10,11] Activation of α7-nAChR by agonist compounds,[12−17] that is, PNU-282987,[13] SEN12333,[14] SSR180711,[15] EVP-6124,[16] and EVP-5141,[17] leads to procognitive and neuroprotective activities. These findings support the role of α7-nAChR agonists in improving cognition when deficits arise.[18−20]

Several 1,2,3-triazole based compounds have been previously characterized as α7-nAChR agonists; however, their subtype selectivity and influence on cognition have not been established in vivo.[21−23] Two promising candidates, IND8 and QND8, selected by optimization of substituted 1,2,3-triazoles as selective and potent α7-nAChR agonists,[23] were evaluated for the cognitive improvement and enhancement in amnesic mouse model. The structures, binding and functional data of IND8, QND8, and PNU-282987, a reference α7-nAChR agonist, are shown in Figure 1. In this study, three models of behavioral testing, that is, modified Y-maze, object recognition test (ORT), and water maze were performed in mice to evaluate these α7-nAChR agonists on cognitive improvements for both short- and long-term memory in scopolamine-induced amnesic mouse model. Moreover, the cognitive enhancement in physiological (natural memory loss) amnesia was assessed using ORT time delay model. Functional antagonism was evaluated to verify that cognitive enhancement was mediated through α7-nAChR activation.

RESULTS AND DISCUSSION

IND8 and QND8 were tested for the cognitive improvement in pharmacologically induced amnesia in mice using three behavioral models. Doses of 5−50 μmol/kg are the range of tested dose for IND8 (1.5−14.7 mg/kg) and QND8 (1.4−13.5 mg/kg), selected from the range of doses reported in behavioral studies of α7-nAChR agonists in animal models,[12−17,24] for example, EVP-5141 (0.3−3 mg/kg),[17] SEN12333 (1−10 mg/kg),[14] and PNU-282987 (10 and 33 μmol/kg or 3 and 10 mg/kg).[12] However, the variation in testing protocols, animal species, and dose regimens employed emphasize the need for multiple cognitive improvement studies and models.

IND8 and QND8 were evaluated for their effects on different types of memory impairment by using mouse models for cognitive deficits. Scopolamine, a muscarinic antagonist, was used to impair cholinergic neurotransmission, shown to elicit deficits in AD patients, and to induce amnesic state in animal and human subjects.[25,26] PNU-282987 was chosen to be a representative of α7-nAChR agonist reference for in vivo profile comparison, because its structure contains the same cationic center (quinuclidine ring), and in vitro and in vivo data have been reported.[12,13,24] PNU-282987, at a dose of 10−33 μmol/kg (3−10 mg/kg), was evaluated for cognitive improvement in two study models, the modified Y-maze and the ORT time delay. These two models reflect different types of memory for

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deficit analysis: spatial working memory for modified Y-maze and episodic short-term memory for ORT. Experiments were divided into two parts, pharmacologically and physiologically induced amnesia. IND8 and QND8 were initially screened to rule out effects on locomotor activity at all tested doses; neither a reduction nor an increase in locomotor activity was observed (Supporting Information Figure S1).

1. Effects on Cognition of Pharmacologically Induced Amnesia. The effect of IND8 and QND8 on short- and long-term memory was evaluated by using modified Y-maze,[27] ORT,[28] and water maze[29] as study models. Data from the scopolamine-treated group (SC) in all experiments indicated that 1 mg/kg of scopolamine can generate amnesia in mice compared with a control group (SH) ($p < 0.05$). Because of its historical significance as an AD drug, tacrine (TC), an acetylcholinesterase inhibitor, was used as a positive reference compound. Pretreatment with 1 mg/kg of tacrine 30 min before scopolamine can improve cognitive deficits induced by scopolamine in all test models ($p < 0.05$); an example is shown in Figure 2.

1.1. Modified Y-Maze.[27] The modified Y-maze test was performed to evaluate spatial working memory. The experiment was divided into three sessions: (i) IND8 at 5, 10, and 50 μmol/kg or 1.5, 2.9, and 14.7 mg/kg doses; (ii) QND8 at 5, 10, and 50 μmol/kg or 1.4, 2.7, and 13.5 mg/kg doses; and (iii) IND8 and QND8 at dose of 25 μmol/kg or 7.3 and 6.8 mg/kg, respectively, and PNU-282987 at 10, 33 μmol/kg or 3, 10 mg/kg doses. There was a significant difference of percentage of unfamiliar arm exploration ($F_{5,48} = 8.065$, $p < 0.001$, $n = 8-10$ mice/group for (i) IND8; $F_{5,52} = 10.301$, $p < 0.001$, $n = 9-10$ mice/group for (ii) QND8; $F_{6,52} = 10.930$, $p < 0.001$, $n = 7-10$ mice/group for (iii) IND8, QND8, PNU-282987). An increase of unfamiliar (novelty) arm exploration, when compared with scopolamine-treated group, indicates that these compounds can compensate for cholinergic deficits and improve spatial working memory. The results are shown in Figure 2. In all three sessions, the unfamiliar arm for exploration of the control group was significantly higher than that of scopolamine-treated group, so the mice were in amnesic state. Tacrine, as well as IND8 in all tested doses (1.5−14.7 mg/kg), apparently reversed the cognitive deficit induced by scopolamine as indicated by higher percentage of novelty-arm exploration than that of the amnesic group ($p < 0.05$, one-way ANOVA with Fisher's LSD post hoc comparison), whereas QND8 improved the cognitive deficit significantly only at the 2.7 mg/kg dose. Hence, a third testing session was conducted at 25 μmol/kg to confirm the response efficacy at high QND8 dose and to compare its potency with the reference PNU-282987. The results showed that both IND8 and QND8 significantly improved cognitive deficits in lower doses than that of PNU-282987.

1.2. Object Recognition Test (ORT).[28] The effect of compounds to improve episodic (nonspatial) short-term memory, that is usually impaired in AD patients, was evaluated by an ORT based on an innate preference of mice to explore a novel object rather than a familiar one. The preference test was initially conducted to indicate that mice did not prefer one object (a familiar in brown cylinder shape) to another one (a new object in green triangular shape). The exploration time between two objects did not differ significantly ($p = 0.35$); thus, there was no preference between familiar and new objects. Therefore, the different exploration time between two objects (familiar vs novel) after treatment refers to the ability of mice to memorize the explored object. Mice with amnesia induced by scopolamine spent equal time to explore novel and familiar objects ($p > 0.05$, paired Student's t test), whereas mice in control group or amnesic mice treated with test compounds exhibited significantly different exploration time between objects (Figure 3), reflecting a significant improvement from amnesic state ($p < 0.05$, paired Student's t test).

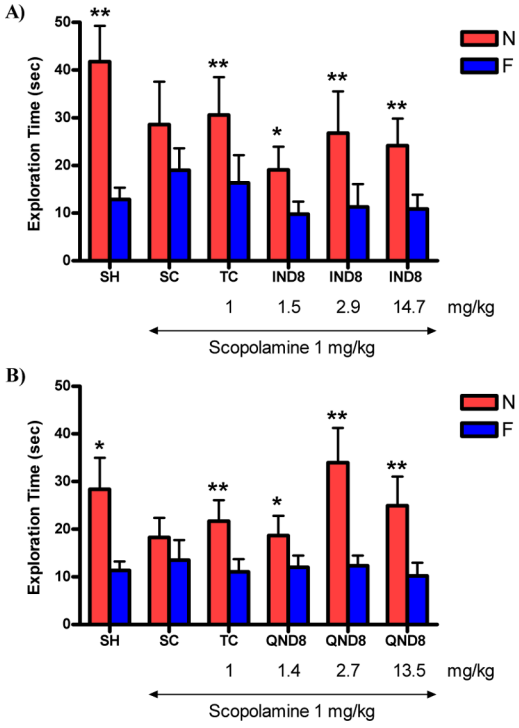

Figure 3. Cognitive improvement in scopolamine-induced amnesia (ORT). Exploration times for familiar (F) and novel (N) objects of IND8 (A) and QND8 (B) in test phase of ORT are shown. On day 1, each mouse was allowed to explore an open field apparatus for 5 min in a habituation phase. After a 24 h period, on day 2, IND8, QND8, vehicle (15% Tween 80, SH), and tacrine (TC) were injected 1 h and scopolamine (SC) 30 min before sample phase, respectively. Each mouse explored two identical objects in the sample phase for 5 min and then was placed in its cage for 10 min before the test phase began. For the 5 min of test phase, one of the objects was changed to a new one. The exploration times directed to these objects were recorded. Data are presented as means ± SE; $^*p < 0.05$, $^{**}p < 0.01$ vs exploration time of familiar object, paired Student's t test.

In both data sets, a discrimination index (DI) was calculated and used to evaluate the ability of mice to discriminate between familiar and novel objects. Data are presented in Figure 4. There was a significant difference of DI ($F_{5,38} = 4.694$; $p = 0.002$; $n = 7-8$ mice/group for IND8 group; $F_{5,44} = 2.900$; $p = 0.024$; $n = 7-9$ mice/group for QND8 group). The higher DI than that of scopolamine-treated group reflects compensation for cholinergic deficits and improvement in episodic short-term memory. The DI of control group was significantly higher than scopolamine-treated group reflecting their ability to discriminate between familiar and novel objects. Tacrine at 1 mg/kg and IND8 and QND8 at 10 and 50 μmol/kg (2.9, 14.7 mg/kg for IND8 and 2.7, 13.5 mg/kg for QND8) significantly improved episodic short-term memory ($p < 0.05$) as monitored by the increase of DI compared with amnesic group ($p < 0.05$, one-way ANOVA with Fisher's LSD post hoc comparison).

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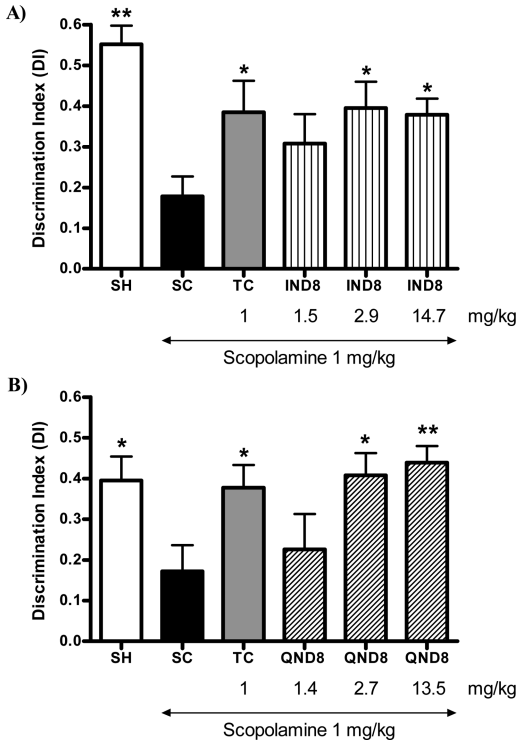

Figure 4. Discrimination index ($T_N - T_F/T_N + T_F$) of IND8 (A) and QND8 (B) in the test phase of ORT. Data are presented as means ± SE; n = 7−8 mice/group for IND8 group; n = 7−9 mice/group for QND8 group. *p < 0.05, **p < 0.01 vs scopolamine-treated group (SC), one-way ANOVA with Fisher's LSD post hoc comparison. SH, control group (vehicle); TC, tacrine group.

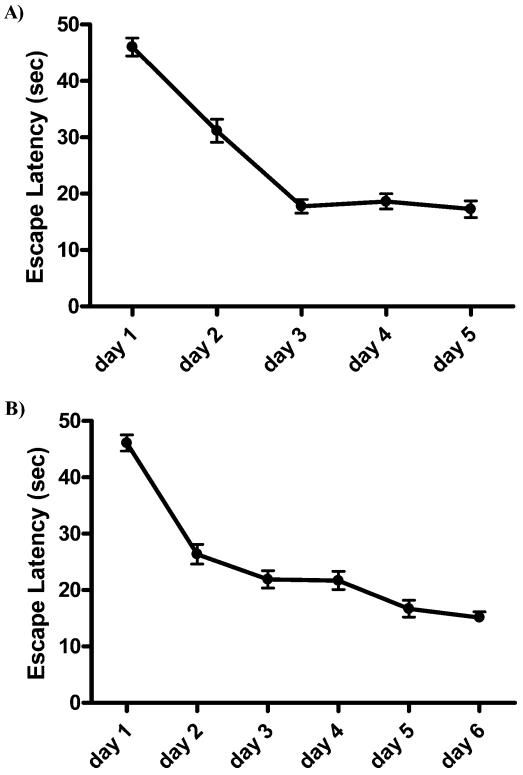

Figure 5. Escape latency time during training period of session A (n = 52) and session B (n = 63).

1.3. Water Maze.[29] The water maze test, a model for spatial learning and reference memory, related to function in the hippocampus, was also used as a test in which mice locate a target quadrant previously placed hidden platform after a training session. Mice were trained to locate a hidden platform until a steady state measured by a shortened escape latency time was reached. During the training period, the escape latency dramatically decreased during the first 3 days and reached the steady state around day 5 as shown in Figure 5.

The average swimming time in a target quadrant where the platform had previously been placed was measured in the test day, day 8 or 9 (Figure 6). Findings with the treatment group of mice, that spent more time in the target quadrant than scopolamine-treated group, indicate spatial memory improvement. There was a significant difference of time spent in the target quadrant ($F_{6,45}$ = 5.079, p < 0.001, n = 7−10 mice/group for IND8 group; $F_{6,56}$ = 2.465, p = 0.035, n = 9 mice/group for QND8 group). The time spent in the target quadrant of control group was significantly higher than that of the scopolamine-treated group. Tacrine significantly improved cognitive deficits indicated by spending more time in the target quadrant than amnesia group. Mice treated with IND8 at 25 and 50 μmol/kg (7.3 and 14.7 mg/kg), and QND8 at 25 μmol/kg (6.8 mg/kg) also spent significantly more time than amnesia group (p < 0.05) in the target quadrant, indicating spatial long-term memory improvement.

The results from the amnesic mouse model induced by scopolamine indicated that IND8 and QND8, α7-nAChR agonists, clearly improved cognitive learning and memory performance in mice with cholinergic deficits. Since the α7-nAChR is involved in memory formation, reversal of scopolamine-induced amnesia may be due to the ability of test compounds as α7-nAChR agonists acting presynaptically to promote the release of acetylcholine or other transmitters either surmounting or compensating for the muscarinic antagonism. Alternatively, direct postsynaptic α7-nAChR activation may be directly stimulatory.[12] However, the pattern of IND8 and QND8 behavioral responses appears to decrease in a higher dose range, similar to reports on other compounds acting in the cholinergic system.[14,30]

2. Effects on Cognition of Delay Induced Memory Deficit.[16] Both compounds were further evaluated on a physiologic amnesic model by using ORT with a 24 h time delay to induce natural memory loss in mice. The appropriate time delay causing amnesia was initially selected by varying delay times (24, 48, and 72 h) between sample and test phases that employ identical and different objects, respectively. The exploration time between two different objects was not significantly different in the test phase after 24, 48, and 72 h of first exploration (p > 0.05, paired Student's t test) as shown in Figure 7. Based on this result, mice did not retain memory of objects for 24 h following the first exploration. Therefore, 24 h was used as the time delay between sample and test phase.

The exploration time between two identical objects (F0 vs F1) of each treatment groups was not significantly different (Figure 8A, p > 0.05 analyzed by Student's t test). The exploration time at only one object (F0) between each treatment groups was not significantly different (p = 0.482, one-way ANOVA), nor were those from the identical object (F) (equal variance test failed; p = 0.496 for one-way ANOVA on ranks). In the test phase, exploration times between novel and familiar objects were significantly different in all treatment groups (p < 0.05) except for mice in control group that

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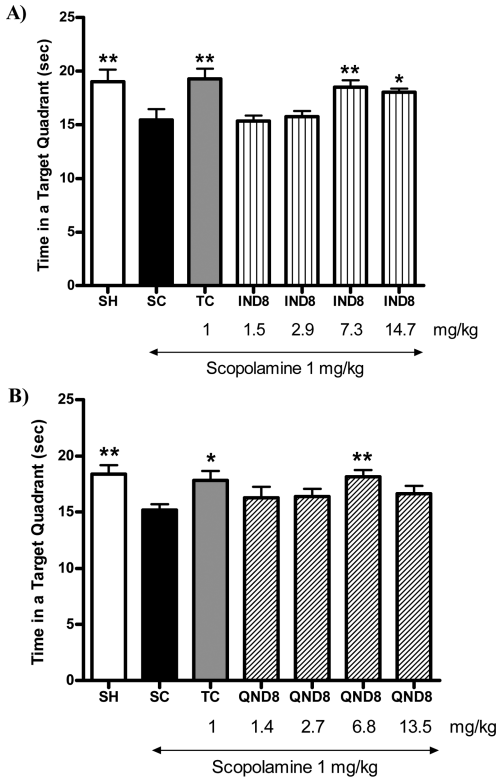

Figure 6. Cognitive improvement in scopolamine-induced amnesia (water maze test). Data are presented as means ± SE of the swimming time in the target quadrant of water maze test. IND8, QND8, vehicle (15% Tween 80, SH), and tacrine (TC) were injected 1 h and scopolamine (SC) 30 min before testing, respectively; n = 7−10 mice/group for IND8 group, n = 9 mice/group for QND8 group, $^{*}p < 0.05$, $^{**}p < 0.01$ vs scopolamine-treated group (SC), one-way ANOVA with Fisher's LSD post hoc comparison.

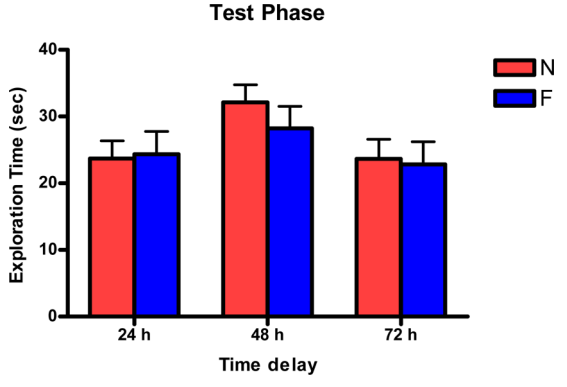

Figure 7. Exploration time for protocol validation. Exploration time delays of the test phase were studied with 24, 48, and 72 h time intervals between sample and test phase. Each mouse was allowed to explore two identical objects in the sample phase for 5 min. After 24, 48, or 72 h time intervals, one of the objects was changed to a different visual shape and color, and mice were allowed to explore these objects for 5 min. Exploration times of these objects were recorded. Data are presented as means ± SE, n = 7−8 mice/group. Paired Student's t test was used to evaluate the different exploration times between novel and familiar objects.

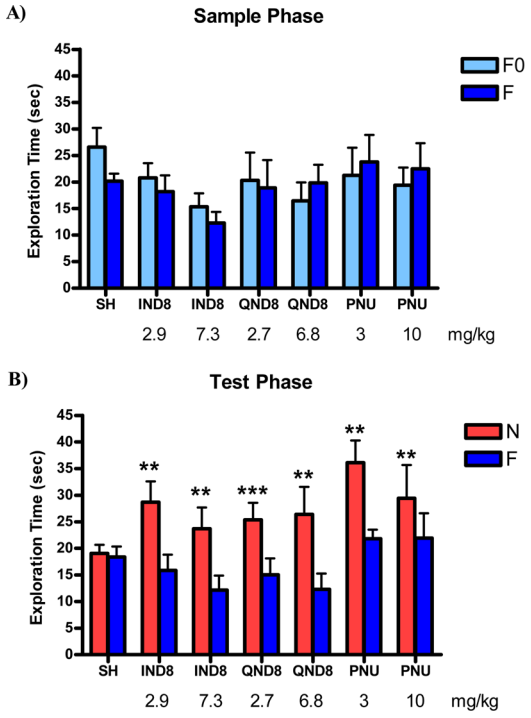

Figure 8. Cognitive enhancement in memory loss after a 24 h time delay. Exploration times to identical objects (F0 and F) in sample phase (A), and familiar (F) and novel (N) objects in test phase (B) of ORT following a time delay were recorded. On day 1, each mouse was allowed to explore an open field apparatus for 5 min in a habituation phase before a sample phase on day 2. On day 2 (sample phase), vehicle (SH) and test compounds were injected 1 h before measurement of exploration time. Each mouse was allowed to explore two identical objects in the sample phase for 5 min. On day 3 (test phase), one of the objects was changed to a new one and the mouse was allowed to explore these objects for 5 min. The exploration time of these objects was recorded. Data are presented as means ± SE, n = 6−8 mice/group. $^{**}p < 0.01$, $^{***}p < 0.001$ vs exploration time of familiar object, paired Student's t test.

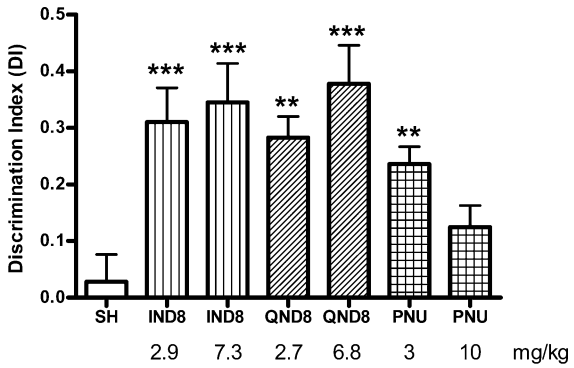

Figure 9. Discrimination index $(T_N - T_F/T_N + T_F)$ of each compound in the test phase of ORT with a time delay protocol. n = 6−8 mice/group; $^{**}p < 0.01$, $^{***}p < 0.001$ vs vehicle group (SH), one-way ANOVA with Fisher's LSD post hoc comparison.

received only vehicle (Figure 8B). The DI was calculated and compared with the control group to evaluate IND8, QND8, and PNU-282987 enhancement of cognitive function. Data are shown in Figure 9. There was a significant difference in DI ($F_{6,43}$ = 6.097, $p < 0.001$, n = 6−8 mice/group). Administration of IND8 and QND8 can enhance cognition with the same pattern as PNU-282987 as monitored by the increase of DI. Mice that received IND8 and QND8 at 10 and 25 μmol/kg (2.9, 7.3 mg/kg for IND8 and 2.7, 6.8 mg/kg for QND8) and PNU-282987 at 3 mg/kg exhibited enhanced cognition ($p <$

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0.05 vs control group, one-way ANOVA with Fisher's LSD post hoc comparison).

3. Mechanism of the Cognitive Enhancement Action. To verify that the mechanism of action of IND8 and QND8 on cognitive enhancement is mediated through α7-nAChRs, IND8 and QND8 at the minimum effective doses from previous experiments, 10 μmol/kg (2.9 and 2.7 mg/kg, respectively), and PNU-282987 10 μmol/kg (3 mg/kg) were evaluated by using a selective α7-nAChR antagonist. Methyllycaconitine (MLA) was injected to block the α7-nAChR agonist response. The ORT in natural memory loss mice described above was the study model. Exploration times between two objects in both sample and test phases of mice receiving MLA was not significantly different; obtaining a pattern similar to vehicle indicates that MLA itself does not enhance episodic memory, as shown in Figure 10. The exploration time between two identical objects in sample phase was not significantly different between all treatment groups (Figure 10A), whereas time to explore between novel and familiar objects was significantly different ($p < 0.05$) in all treatment groups of test compound alone (Figure 10B). The higher bars of the second pair than the first pair in Figure 10B is due to a transient uncontrolled variable or random variation since the red bars of the first two pairs are not significantly different (t test, $p = 0.23$) nor are the blue bars ($p = 0.19$). As expected, the exploration times between novel and familiar objects of control group and the MLA pretreatment groups were not significantly different. These results indicated that IND8 and QND8 mediated the episodic memory enhancement through α7-nAChRs, since MLA is a selective α7-nAChR antagonist.

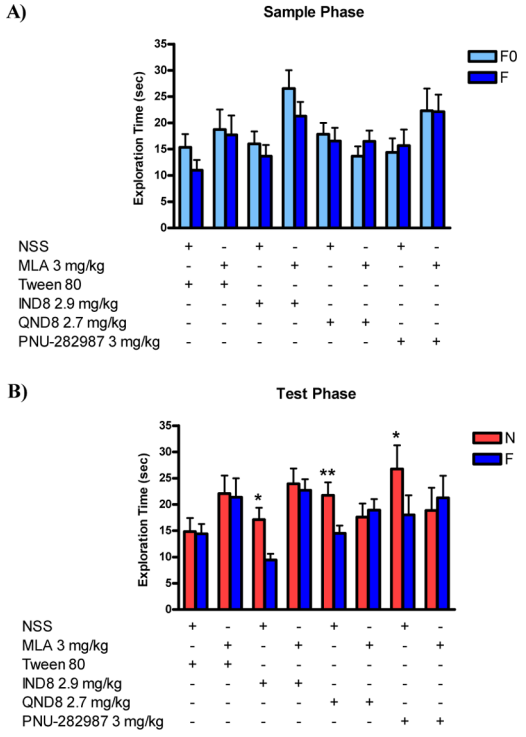

Figure 10. Exploration times to identical objects (F0 and F) in sample phase (A), and familiar (F) and novel (N) objects in test phase (B). ORT is used to establish that the mechanism of action is mediated through α7-nAChRs. On day 1, each mouse was allowed to explore an open field apparatus for 5 min in a habituation phase. After 24 h on day 2 (sample phase), MLA (3 mg/kg) was injected 5 min before the test compounds. One hour after injection of the test compounds, each mouse explored two identical objects for 5 min. On day 3 (test phase), one of the objects was changed to a new one and the mouse was allowed to explore these objects for 5 min. The exploration time of these objects was recorded. Data are presented as mean ± SE, n = 7–10 mice/group. $^{**}p < 0.01$, $^{***}p < 0.001$ vs exploration time of familiar object, paired Student's t test.

Evaluating the DI, mice receiving vehicle did not recognize the object that they had explored in the sample phase and MLA alone did not exhibit cognitive enhancement; therefore, the DI in both groups was near zero. Our results confirmed the previously reported effect of MLA that MLA does not enhance memory,[15,31] rather it may impair memory.[32,33] For IND8, QND8, and PNU-282987, the DI's in these three groups were significantly higher than that of control group which indicated memory enhancement in mice ($p < 0.05$ vs control group, one-way ANOVA with Fisher's LSD post hoc comparison), as shown in Figure 11. This cognitive enhancement was abolished by MLA, where the DI of MLA pretreated group returned to the same value as vehicle or MLA groups. The basis for MLA action could arise from direct cognitive impairment by the antagonist itself or competitive antagonism of the test α7 agonists. However, the previously reported impaired recognition test occurred after a 24 h interval.[32] In addition, the reported MLA induced cognitive deficit was associated with mice hypermotility in T-maze study.[33] These differences in time interval of administration and the lack of detected hypermotility in our study indicate that blockade of cognitive enhancement elicited by IND8, QND8, and PNU-282987 is mediated through antagonism of the test agonists on α7-nAChR as seen in the DI differences with and without MLA.

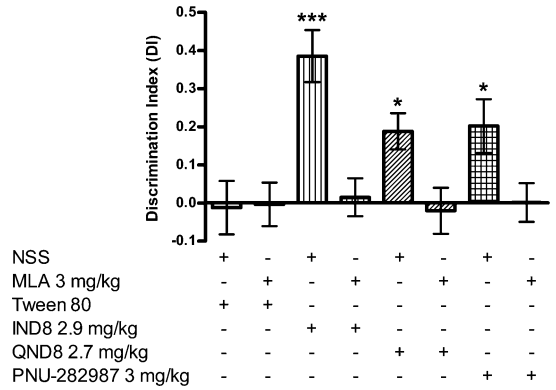

Figure 11. Discrimination index ($T_N - T_F/T_N + T_F$) of each compound in the test phase of ORT for establishing a mechanism of action, $^{**}p < 0.01$, $^{***}p < 0.001$ vs vehicle group, one-way ANOVA with Fisher's LSD post hoc comparison.

From in vivo results, IND8 and QND8 (5 and 10 μmol/kg, respectively) have higher potency than PNU-282987 (33 μmol/kg) for the improvement of spatial working memory as observed in scopolamine-induced cognitive deficit mice in modified Y-maze test. The same potency of IND8 and QND8 (10 μmol/kg) required to improve episodic short-term memory and reference long-term memory in cognitive deficit mice was observed in ORT and water maze test. Moreover, these α7-nAChR agonists, IND8, QND8, and PNU-282987, are able to enhance cognitive function that was evaluated in ORT following a time delay to induce amnesia. The minimum effective dose data reflect the relative functional potencies seen

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Table 1. In Vitro Agonist Binding Properties and in Vivo Efficacy

			minimum effective dose (μmol/kg)			
compd	K_d ± SD (μM)[a]	EC_{50} ± SD (μM)[a]	modified Y-maze[b]	ORT[b]	water maze[b]	ORT[c]
PNU-282987	0.27 ± 0.14	0.11 ± 0.01	33	n.d.	n.d.	10
IND8	0.12 ± 0.06	0.028 ± 0.010	5	10	25	10
QND8	0.081 ± 0.029	0.037 ± 0.009	10	10	25	10

[a]K_d and EC_{50} for α7-nAChR agonist from radioligand binding assay and functional assays using cells expressing LGIC receptors.[23] [b]Pharmacologically (scopolamine) induced amnesia model. n.d., not determined. [c]Physiologically induced amnesia (memory retention) model

Table 2. Predicted Drug-Likeness Properties of IND8, QND8, and PNU-282987[a]

properties	parameter	IND8	QND8	(*R*) PNU-282987	recommended range
human serum protein binding	logKhas	0.35 (*R*), 0.36 (*S*)	0.00 (*R*), 0.01 (*S*)	−0.00	−1.5−1.5
brain/blood partition coefficient	logBB	0.20 (*R*), 0.19 (*S*)	−0.08 (*R*), −0.09 (*S*)	0.68	−3.0−1.2
Caco-2 cell permeability	P Caco-2	390[b]	218[b]	1069	<25 poor, >500 great
cell permeability	P MDCK	198[b]	106[b]	1448	<25 poor, >500 great
CYP inhibition	CYP isoform	2D6	2D6	2D6, 2C19	
K^+ channels blockage	logHERG	−5.73	−5.18	−5.10	> −5

[a]In silico prediction by QikProp version 4.1 and WhichCYP version 1.2: logKhas, human serum protein binding; logHERG, $logIC_{50}$ for HERG K^+ channels blockage; logBB, brain/blood partition coefficient; P Caco-2, Caco-2 cell permeability in nm/s; P MDCK, MDCK cell permeability in nm/s; CYP isoform, cytochromes P450 isoform inhibition. (*R*) for R-configuration, (*S*) for S-configuration. [b]The predicted values are the same for both (*R* and *S*) enantiomers.

in the cellular assays for α7 nAChR activity as summarized in Table 1.

4. In Silico Drug Disposition Predictions. Toxicity and pharmacokinetic parameters for blockade of a human ether-a-go-go related gene (hERG) K^+ channel, serum albumin binding, permeability properties, and cytochrome P450 inhibition of IND8, QND8, and PNU-282987 α7-nAChR agonists were predicted in silico by QikProp version 4.1[34] and WhichCYP version 1.2.[35] The in silico prediction points out that these compounds have properties that likely enable them to be absorbed orally, pass the blood-brain barrier, and are likely not rapidly metabolized. The predicted values of all compounds and the recommended range of drug similarity are shown in Table 2. The predicted values of all permeation parameters, that is, brain/blood partition coefficient, Caco-2 cell permeability, and cell permeation MDCK values parallel their physiochemical properties and pK_a values. Although IND8 and QND8 have diminished unprotonated species based on their respective pK_a values of 9.17 and 8.93, they still have moderate intestinal (P Caco-2) and BBB permeability (P MDCK and logBB) when compared to PNU-282987 with its lower pK_a value of 7.86. IND8 and QND8 may inhibit CYP450 isoform 2D6, whereas PNU-282987 probably inhibits two isoforms, CYP2D6 and CYP2C19. IND8 has enhanced likelihood to bind to plasma protein and block hERG K^+ channels compared to PNU-282987 and QND8. As PNU-282987 showed 57% inhibition of hERG at 20 μM in a patch clamp hERG K^+ channel assay,[36] these two optimized lead compounds should be evaluated in vitro for ion channel blockade together with other preclinical assays, such as CYP inhibition, multidrug resistant (MDR), and P-glycoprotein (PgP) assays.

IND8 and QND8 differ from PNU-282987 where their linkage to a second aromatic ring is through a 1,4-substitution of a 1,2,3-triazole, instead of an amide. With the initial behavioral and binding parameters, it may be possible to refine further the structure−activity relationships, based on crystal structure determination of the respective complexes. Triazoles form suitable linkers in that *syn*- and *anti*-regioisomers can be generated with Ru^+ and Cu^+ catalysis.[37] The triazole linker possesses other advantages, since the efficiency of the metal-catalyzed reactions in the absence of a template enables reaction completion for small building block quantities in a combinatorial and in an arrayed fashion. Only the leads then need to be generated in the several milligrams to gram level for in vivo studies. The minor drawbacks for click synthesis are scale-up difficulties due to its highly exothermic nature and the explosive potential of certain azides.

Taking in vitro and in vivo results together, IND8 and QND8 have higher potencies than PNU-282987, even though the drug-disposition data predicted in silico suggests slower rates of brain penetration of IND8 and QND8 than that of PNU-282987 due to lower P MDCK and logBB and higher pK_a values. A possible reason for this is that the protonated quinuclidine is the active species upon equilibration forming a hydrogen bond donor with the backbone carbonyl oxygen of conserved Trp 149 in the α7-nAChR.[38]

■ CONCLUSIONS

IND8 and QND8, novel potent α7-nAChR agonists, derived and refined from in situ click-chemistry synthetic leads, can reverse amnesia and improve spatial working memory, episodic short-term memory, and reference long-term memory, all behavioral parameters typically impaired in AD. Moreover, they appear to enhance cognition. Based on correlations between occupation and activation of α7-nAChRs in intact cells and antagonism of the behavioral response by MLA, this enhancement is mediated through α7-nAChRs. The physical characteristics of the substituted triazoles bode well for crossing the blood-brain barrier and being retained in the CNS. Hence, these findings support the potential of IND8 and QND8 and other substituted 1,2,3 *syn*- and *anti*-triazole analogues as preclinical candidates in treatment of cognitive disorders.

■ METHODS

1. In Vivo Assay. *1.1. Animal Model.* Male ICR mice (6-weeks of age) from National Laboratory Animal Center, Mahidol University, Thailand were housed in five per cage with free access to food and tap

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ACS Chem. Neurosci. 2015, 6, 1331−1340

water under 12 h light and dark cycle (light on 6:00 to 18:00) in a temperature and humidity controlled room. They were acclimated in a laboratory at least 7 days prior to starting experiments. The experimental protocols were approved by the Animal Care and Use Committee of KhonKaen University, Thailand (Record No. AEKKU 34/2557, Reference No. 0514.1.12.2/38).

1.2. Compound Preparation and Administration. Tacrine hydrochloride (Sigma-Aldrich, St. Louis, MO), PNU-282987 hydrochloride (Alamone, Jerusalem, Israel), and test compounds were prepared as suspensions by using 15% Tween 80 in distilled water as a vehicle, whereas scopolamine hydrochloride (Sigma-Aldrich, St. Louis, MO) and methyllycaconitine citrate (MLA) (Abcam, Cambridge, England) were prepared as a solution by dissolving with 0.9% normal saline solution (NSS). All compounds were administered by intraperitoneal (i.p.) injection in a volume of 5 mL/kg. Test compounds were injected 1 h and scopolamine was administered 30 min before the behavioral experiments, whereas MLA was injected 5 min before the test compounds.

1.3. Determination of Scopolamine-Induced Cognitive Deficit Improvement. Amnesia was induced pharmacologically in mice via scopolamine injection (single dose) for 30 min prior to start of the experiments. They were divided into a (i) vehicle group, receiving 15% Tween 80 and 0.9% NSS, (ii) amnesia group, receiving scopolamine 1 mg/kg, (iii) standard positive control group, receiving tacrine 1 mg/kg, and (iv) test group, receiving IND8, QND8 at dose 5, 10, 25, 50 μmol/kg, or PNU-282987 3, 10 mg/kg, single dose. All mice were acclimated in an experimental room at least 30 min before the experiments.

1.3.1. Locomotor Activity Test. A black polyvinyl chloride Y-maze, which was 40 cm long, 3 cm wide at the bottom and 10 cm wide at the top, and 12 cm high in each arm, was used to study the influence of synthesized compounds on locomotor activity. Each mouse was placed at the end of one arm and was allowed to move freely in Y-maze for 8 min 1 h after injection of vehicle or test compounds. The number of entries to all arms was recorded visually if mice accessed each arm at least 10 cm from the middle of the maze.

1.3.2. Modified Y-Maze Test.[27] The maze in this experiment is the same apparatus used in locomotor activity tests except for a black partition used to close one of three arms. The mice were randomly separated into different treatment groups. They were injected with test compounds and scopolamine at 1 h and 30 min, respectively, before the sample phase. In this phase, where one arm of Y-maze was closed by the black partition, each mouse was placed at the end of one arm and allowed to move through two arms for 5 min to get familiar with the two open arms. After 30 min of resting, all arms were opened and the mouse was allowed to move freely through all three arms. The maze area and the partition were cleaned with 70% ethanol between each experiment to remove olfactory cues. The number of entries in each arm was counted visually if mice accessed each arm at least 10 cm. The percentage of unfamiliar arm exploration was calculated as following equation:

Mice that did not leave the arm they were placed in were excluded from the data analysis.

$$\text{percentage of unfamiliar arm exploration} = \frac{\text{number of unfamiliar arm entry}}{\text{number of all arms entry}} \times 100$$

1.3.3. Novel Object Recognition Test (ORT).[28] The apparatus was made of black polyvinyl chloride (52 × 52 × 40 cm^3). The objects used in this experiment had different visual shapes and colors to be discriminated. One object (familiar object) is a brown cylinder shape, whereas another one (new object) is a green triangular shape, made of glass and plastic, respectively. They were placed 10 cm from the side wall in the balanced manner. The box area and objects were cleaned with 70% ethanol between each experiment to remove odor cues.

The mice were randomly divided into different treatment groups. On day 1, they were allowed to freely explore the open field apparatus for 5 min for habituation in the day prior to the experiments to get familiar with the apparatus. On day 2, test compounds were injected 1 h, whereas scopolamine was administered 30 min before the sample phase (acquisition trial). The mice were placed in the apparatus to explore two identical objects. After 10 min of acclimation in their cages, one object was changed to a new one, and mice were allowed to explore these objects for 5 min in the test phase (retention trial). The exploration time of each object was recorded if the nose of mice approached the objects within 3 cm or touched the objects. The discrimination index (DI) was calculated by using $(T_N - T_F)/(T_N + T_F)$; T_N and T_F represented exploration time of new and familiar objects, respectively. Mice that always stayed at the corner of the apparatus had less than 5 s of total exploration time during the sample phase,[39] or explored only one object in sample phase were excluded from the data analysis.

1.3.4. Water Maze.[29] A black circular tank (diameter 70 cm; height 28 cm) was divided into 4 quadrants with a removable escape platform (6 × 7.5 × 14 cm) centered in a target quadrant. The tank was filled with water to 15 cm height and the platform was located 1 cm below the water surface.

Mice were trained from 5 to 6 days to reach steady state of escape latency. Mice were released from all quadrants except in probe test and for the test day when the target quadrant was not used as the release point. On the training day (day 1 to 5 or 6), each mouse was trained to swim by release in each quadrant for a total of four trials with 1 min maximum time and allowed to stay on the platform for 10 s in each trial. The mouse is placed on the platform for 10 s if it cannot find the hidden platform within 1 min. The escape latency time from each quadrant was measured and averaged. Training sessions were ended when the escape latency time does not significantly differ from the former day. At the end of training session, around 7−8% of the mice that cannot locate the platform and do not show a learning ability measured by a decrease of escape latency time were excluded. After reaching steady state, the probe test began and the platform was removed. For the probe test, there were three trials released from three quadrants excluding the target quadrant that had been placed the platform. Time spent in the target quadrant of all three trials was measured and averaged. On the day after probe test, the platform was brought back to remind the mice of the target quadrant location. On the next day (test day), all conditions were the same as probe test, but mice were injected with test compounds 1 h and scopolamine 30 min before testing. The swimming time in the target quadrant was measured and averaged. Mice that jumped out of the tank or exhibited persistent floating were excluded from the data analysis.

1.4. Determination of Cognitive Enhancement through α7-nAChRs.[16] The apparatus used in this experiment was the same as that in the ORT test. This experiment was divided into two parts directed to dose finding and establishing mechanism of action. Physiologically induced amnesia (natural temporal memory loss) was passively achieved with a 24 h time interval.

For dose finding protocol, the mice were randomly divided into different treatment groups, a control group receiving a vehicle and tested groups receiving PNU-282987 at 3, 10 mg/kg, and IND8, QND8 for 10, 25 μmol/kg, single dose. They were allowed to freely explore the open field apparatus for 5 min for habituation in a day before the experiment (day 1). On day 2 (the sample phase), the test compound was injected 1 h before the sample phase. In this phase, mice were placed in the apparatus to explore two identical objects. On day 3 (the test phase), one object was changed to a new one and mice were allowed to explore these objects for 5 min in the test phase. To establish cognitive enhancement mediated through α7-nAChRs, the protocol was the same as mentioned above except MLA (3 mg/kg), a selective α7-nAChR antagonist, was injected 5 min before test compounds in the sample phase (day 2) to block the cognitive enhancement from test compounds. The dose of test compounds is selected from the minimum effective dose from the dose finding experiment. The exploration time of each object was recorded if the nose of mouse came within 3 cm or touched the objects. The discrimination index (DI) was calculated by using $(T_N - T_F)/(T_N + T_F)$; T_N and T_F represented exploration time of new and familiar objects, respectively. Exclusion criteria are the same as ORT study determining scopolamine-induced cognitive deficit improvement.

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ACS Chem. Neurosci. 2015, 6, 1331−1340

1.5. Statistics. All results were represented as means ± standard error (SE) for each data group. Statistics were analyzed by using SigmaStat32. Differences of $p < 0.05$ were considered significant.

The different results from the number of entries for locomotor activity testing between test and vehicle groups were analyzed with one-way ANOVA, followed by Fisher's least significant difference (LSD) post hoc comparison.

For determination of scopolamine-induced cognitive deficit improvement in modified Y-maze, ORT, and water maze, the results between test groups and amnesia group were analyzed with one-way ANOVA, followed by Fisher's LSD post hoc comparison, except the exploration time between sample and test phase for ORT was analyzed by a paired Student's *t* test.

For determination of cognitive enhancement through α7-nAChRs, one-way ANOVA, followed by Fisher's LSD post hoc comparison was used to compare the DI between test and vehicle groups in a dose finding protocol and compared with and without MLA administration to establish a mechanism of action. Exploration times between sample and test phase were analyzed by paired Student's *t* test.

2. In Silico Prediction. Structures of IND8 and QND8 were prepared as neutral species for the stereoisomers by LigPrep version 3.1.[40] The physiochemical and pharmaceutical properties of prepared ligands were predicted by QikProp version 4.1.[34]

■ ASSOCIATED CONTENT

Supporting Information

Dissociation constants (K_d) and functional properties (Table S1); locomotor activity testing (Figure S1). The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.5b00059.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AD, Alzheimer's disease; BBB, blood−brain barrier; DI, discrimination index; FRET, fluorescence resonance energy transfer; hERG, human ether-a-go-go related gene; i.p., intraperitoneal; MDCK cells, Madin-Darby canine kidney cells; LGIC, ligand-gated ion channel; LSD, least significant difference; MLA, methyllycaconitine; nAChR, nicotinic acetylcholine receptor; NSS, normal saline solution; ORT, object recognition test; SD, standard deviation; SE, standard error; LGIC ligand-gated ionic channel; ICR, imprinting control region

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