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Current Status of Antisense DNA Methods in Behavioral Studies

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Abstract

The antisense DNA method has been used successfully to block the expression of specific genes *in vivo* in neuronal systems. An increasing number of studies in the last few years have shown that antisense DNA administered directly into the brain can modify various kinds of behaviors. These findings strongly suggest that the antisense DNA method can be used as a powerful tool to study causal relationships between molecular processes in the brain and behavior. In this article we review the current status of the antisense method in behavioral studies and discuss its potentials and problems by focusing on the following four aspects: (i) optimal application paradigms of antisense DNA methods in behavioral studies; (ii) efficiencies of different administration methods of antisense DNA used in behavioral studies; (iii) determination of specificity of behavioral effects of antisense DNA; and (iv) discrepancies between antisense DNA effects on behaviors and those on protein levels of the targeted gene.

Introduction

The antisense DNA/RNA method has been growing in status, in the last few years, as a new and powerful tool for the study of molecular bases of behaviors. A number of studies from this laboratory (Ogawa *et al.*, 1992, 1994; McCarthy *et al.*, 1993, 1994a,b; Kow *et al.*, 1996; Nicot *et al.*, 1997) and many others have shown that antisense DNA administered directly into the brain can modify the occurrence of various kinds of behaviors by blocking the synthesis of the targeted gene products of *in vivo* neuronal systems (for reviews see Ogawa and Pfaff, 1996; Wahlestedt, 1994). The antisense method, involving active manipulation of gene expression, can more directly demonstrate the role of certain molecular processes in the regulation of a specific behavior than can correlational studies. In addition, the antisense method has some advantages over transgenic animals, another new and powerful molecular tool for behavioral studies: (i) the antisense method can be applied to any gene product of any species as long as genetic sequence information is available; (ii) blockade of the gene expression by the antisense method is reversible (e.g. manipulation of targeted gene products at a specific developmental stage is possible); and (iii) local manipulation (e.g. specific brain regions) of gene expression is possible with the antisense method.

The purpose of this article is to review the current status of the antisense method (mainly the antisense DNA method) in behavioral studies and discuss its potential and problems by focusing on the following four aspects: (i) optimal application paradigms of antisense DNA methods in behavioral studies; (ii) efficiencies of different administration methods of antisense DNA used in behavioral studies; (iii) determination of specificity of behavioral effects of antisense DNA; and (iv) discrepancies between antisense DNA effects on behaviors and those on protein levels of the targeted gene.

Optimal application paradigms of antisense DNA methods in behavioral studies

It is possible theoretically to target any gene product by antisense DNA as mentioned above. Many of the currently reported successful application studies, however, are aimed at blocking agonist action by pretreatment with antisense oligodeoxynucleotides (ODNs) for mRNA of specific receptors, including steroid hormones, neurotransmitters and neuropeptides. Studies in this laboratory have shown that intrahypothalamic administrations of antisense ODNs for estrogen receptor mRNA (McCarthy *et al.*, 1993b) and progesterone receptor mRNA (Ogawa *et al.*, 1992, 1994) can interfere with behavioral effects of testosterone (which acts through estrogen receptors after being aromatized to estradiol) and progesterone, respectively, on female sexual behaviors. Pretreatment with antisense ODNs for either D_1- or D_2-dopamine receptor mRNA can inhibit locomotor or grooming behaviors induced by specific D_1- or D_2-dopamine receptor agonists, but not vice versa (Weiss *et al.*, 1993; Zhang and Creese, 1993; Silvia *et al.*, 1994; Zhang *et*

al., 1994; Zhou *et al.*, 1994). It has also been reported that intrathecal or intracerebroventricular administrations of antisense ODNs to δ or κ opioid receptor selectively inhibited δ or κ opioid receptor agonist-induced antinociception for the relevant receptor without affecting analgesia induced by agonists of other receptor subtypes (Chien *et al.*, 1994; Lai *et al.*, 1994; Standifer *et al.*, 1994). Modulation of angiotensin II-induced drinking behaviors by pretreatment with antisense ODNs to type 1 angiotensin receptor mRNA has also been reported (Sakai *et al.*, 1994). In addition to antisense ODNs for mRNA of various receptors, pretreatment with antisense ODNs for the immediate early gene, *c-fos*, appears to block rotational and locomotor behaviors induced by various agonists [e.g. amphetamine or cocaine (Dragunow *et al.*, 1993; Heilig *et al.*, 1993; Sommer *et al.*, 1993)].

It should also be noted that relatively large and consistent behavioral effects after a single (or at most two) injection of antisense ODNs with a short latency are often reported in the studies targeting the synthesis of inducible gene products, in contrast to those targeting the synthesis of constitutively active gene products. For example, in ovariectomized rats and mice, it has been well described that both mRNA and protein levels of progesterone receptors are induced in specific hypothalamic regions in a time-dependent manner after subcutaneous estrogen injection (Parsons *et al.*, 1980; Romano *et al.*, 1989; Ogawa *et al.*, 1993). By injecting antisense ODNs for progesterone receptor mRNA at 12 h after estrogen injection, we could inhibit both the progesterone receptor-mediated facilitatory action of progesterone on female sexual behaviors and progesterone receptor immunoreactivity in rats (Ogawa *et al.*, 1992, 1994), as well as progestin binding levels in mice (Ogawa and Pfaff, 1996) at 48 h after estrogen injection. Similar behavioral effects of antisense ODNs for progesterone receptor mRNA are also reported by two other groups (Pollio *et al.*, 1993; Mani *et al.*, 1994a,b). Likewise, a single pretreatment (5.5–10 h prior) with antisense ODNs for c-fos mRNA inhibits rotational and locomotor behaviors induced by amphetamine or cocaine as well as causing almost complete disappearance of amphetamine- or cocaine-inducible c-fos-like immunoreactive cells (Chiasson *et al.*, 1992; Dragunow *et al.*, 1993; Heilig *et al.*, 1993; Sommer *et al.*, 1993).

In some cases, however, the antisense method has been successfully used to modify behavior by blocking endogenous peptide synthesis. For example, Akabayashi *et al.* (1994) have shown that antisense ODNs for neuropeptide Y mRNA reduced peptide synthesis in neurons in the arcuate nucleus and also reduced carbohydrate and fat intake. It should be noted that in this study the antisense method was used as an alternative method because a specific inhibitor of peptide synthesis is not available to provide direct evidence of the involvement of neuropeptide Y in the regulation of feeding behaviors.

As described above, one of the major advantages of the antisense method as opposed to knockout transgenic animals is that the blockade of gene expression by the antisense method is reversible. The antisense method, therefore, can be a powerful tool to study the role of specific molecular processes in neural and behavioral development by treating animals with antisense ODNs at certain developmental stages. For example, McCarthy *et al.* (1993b) have reported that pretreatment with antisense ODNs for estrogen receptor mRNA prior to testosterone injection into neonatal female rats blocks the masculinizing effects of testosterone and results in permanent alteration of behaviors. Similarly, it has been shown that *in vivo* application of antisense ODNs to synaptosomal-associated protein 25 (SNAP-25) mRNA can affect axonal growth of amacrine cells in developing chick retina (Osen-Sand *et al.*, 1993).

Comparisons of efficiencies of antisense ODN administration methods used in behavioral studies

A number of studies in both rats and mice have shown that very little ODN is detected in brain tissue after intravenous or intraperitoneal injections (Agrawal *et al.*, 1991; Iversen, 1991; Zendegui *et al.*, 1992; Cossum *et al.*, 1993; Lu *et al.*, 1994). In contrast, after a single site-specific intracerebral injection into rat brains, ODNs are rapidly taken up by many cells around the injection site in the striatum (Sommer *et al.*, 1993) or in the hypothalamic ventromedial nucleus (McCarthy *et al.*, 1993a). Using either tetramethylrhodamine-5- (and -6)-isothiocyanate (TRITC)- or [γ-^{33}P]ATP-labeled ODNs (15-mer), we have also found that many labeled cells can be detected as early as 5 min after the injection of either labeled phosphodiester oligonucleotides (D-ODNs) or phosphorothioate oligonucleotides (S-ODNs) into mouse brains at the dorsal to the ventromedial nucleus of the hypothalamus (Ogawa *et al.*, 1995). Rostro-caudal and medial–lateral diffusion extended ~500 μm while a relatively large dorsal diffusion along the infusion needle track was often observed. Confocal microscopy 1 h after the infusion confirmed that TRITC-labeled ODNs were indeed inside the cell. Furthermore, most labeled S-ODNs were found in neuronal cells (identified by immunocytochemistry for neurofilament) and to a much lesser extent in astrocytic cells (identified by immunocytochemistry for glial fibrillary acidic protein). These findings provide supportive evidence for large and persistent behavioral effects by a single or relatively small number of repeated bolus intracerebral injections of antisense ODNs (although a large number of repeated injections is still required for constitutively active gene products). To date, site-specific intracerebral injections of antisense ODNs to the striatum (Dragunow *et al.*, 1993; Sommer *et al.*, 1993), the nucleus accumbens (Heilig *et al.*, 1993), the hypothalamic ventromedial (Pollio *et al.*, 1993; McCarthy *et al.*, 1994a,b; Ogawa *et al.*, 1994; Ogawa and

Pfaff, 1996), arcuate (Akabayashi *et al.*, 1994) and supraoptic nuclei (Neumann *et al.*, 1994) and the midbrain central gray (McCarthy *et al.*, 1994b) have been shown to result in reduction of targeted gene products and/or modification of behaviors of interest. It should be noted that with site-specific intracerebral administration it is possible to achieve more precise local manipulation of gene expression by antisense ODNs. In our studies using antisense D-ODNs for progesterone receptor mRNA in both rats and mice, site-specific intracerebral administration of ODNs to the hypothalamic ventromedial nucleus resulted in reduction of estrogen-induced progesterone receptor-like immunoreactivity (Ogawa *et al.*, 1994) or progestin binding (Ogawa and Pfaff, 1996) in this targeted brain region but not in the medial preoptic area. These results are consistent with our observations regarding the range of diffusion of intracerebrally administered ODNs revealed by using TRITC- or [γ-^{33}P]ATP-label as mentioned above (Ogawa et al., 1995).

Intracerebroventricularly (ICV) administered ODNs are also taken up by brain cells (Whitesell *et al.*, 1993; Wahlestedt, 1994). After a single bolus ICV injection of fluorescein-labeled ODNs into rat brain, however, labeled cells can be detected only in ependyma and ODN uptake was markedly decreased away from the ependymal surfaces (Whitesell *et al.*, 1993). Continuous ICV infusion of ODNs with an osmotic minipump for a week leads to a wider distribution of labeled cells. With a bolus injection of TRITC-labeled S-ODNs into the lateral ventricles of mouse brains, we also observed poor tissue penetration of ODNs. These findings suggest that either continuous or many repeated ICV injections of antisense ODNs may be necessary to maximize behavioral effects and especially to block the synthesis of constitutively active gene products. In fact, D_1-dopamine receptor agonist-induced grooming behavior was significantly inhibited by D_1-dopamine receptor mRNA antisense S-ODNs after 10 injections and even more profoundly after 14 ICV injections (twice daily) to mouse brain, but not after three injections (Zhang *et al.*, 1994). In rats, it is also shown that after 3 days of continuous ICV infusions of antisense S-ODNs for D_2-dopamine receptor mRNA, D_2-dopamine receptor agonist-induced locomotor behavior was inhibited (Zhang and Creese, 1993). In contrast, antisense S-ODNs for progesterone receptor mRNA can almost completely block progesterone -induced sexual behaviors in estrogen-primed female rats after two daily ICV injections to the third ventricle (Mani *et al.*, 1994a,b). This relatively large behavioral effect could be due to the fact that progesterone receptor is solely induced by estrogen in a time-dependent manner rather than constitutively expressed as with dopamine receptors.

Finally, intrathecal injections of antisense ODNs also have been successfully used (Chien *et al.*, 1994; Standifer *et al.*, 1994).

One limiting factor of the antisense DNA method is the rapid degradation of oligonucleotides by endogenous exo- and endonucleases (Akhtar *et al.*, 1991b, 1992; Leonetti *et al.*, 1991). As extensively studied for *in vitro* or *in vivo* non-neuronal application of antisense ODNs, it is important to extend the half-life of applied ODNs at the relevant site in the brain to obtain large and persistent behavioral effects. One possible solution is to use chemically modified ODNs. Among many modified oligonucleotides developed to date (Crooke, 1991), S-ODNs, in which one of the oxygens in the internucleotide linkage is substituted with a sulfur atom, are most widely used as an alternative to the unmodified D-ODNs. S-ODNs are known to exert greater effects with much lower concentrations than D-ODNs because they have higher intracellular stability without losing their susceptability to RNase H (Stein *et al.*, 1988, 1991; Zon and Geiser, 1991; Ghosh *et al.*, 1993). For *in vivo* application, S-ODNs are also more stable than D-ODNs after both ICV and intracerebral administration. It has been reported that after bolus ICV injections to the lateral ventricle of rat brain, D-ODNs are quickly degraded in CSF (Whitesell *et al.*, 1993) even though it is stable in CSF *in vitro* (Whitesell *et al.*, 1993; Wahlestedt, 1994). In our intracerebral infusion study we also have found that intense fluorescent signals in cell bodies can be observed for much longer periods of time (up to 8–16 h) after administration of TRITC-labeled S-ODNs than of TRITC-labeled D-ODNs (up to 2–4 h).

The other factor which can improve the cellular uptake and intracellular stability of ODNs is the carrier vehicle. Cationic liposomes, which have been used widely for cellular DNA or RNA transfection (Felgner *et al.*, 1987; Felgner and Ringold, 1989; Jiao *et al.*, 1992), are now recognized as potential carriers of antisense ODNs (Clarenc *et al.*, 1993; Felgner *et al.*, 1994; Thierry *et al.*, 1992; Zhu *et al.*, 1993). Formation of an ODN–liposome complex through ionic interactions enhances cellular uptake of ODNs and affect the intracellular distribution of ODNs in many *in vitro* systems (Akhtar *et al.*, 1991a; Chiang *et al.*, 1991; De Smidt *et al.*, 1991; Mirabelli *et al.*, 1991; Bennett *et al.*, 1992; Thierry and Dritschilo, 1992; Capaccioli *et al.*, 1993). It should be noted, however, that cellular uptake of liposomes through endocytosis leads to association of ODN–liposome complexes with lysosomes and may result in accelerating the degradation of ODNs by lysosomal enzymes [see Ropert *et al.* (1993) for pH-sensitive liposomes that avoid lysosomal degradation]. In our preliminary studies it appeared that with cationic lipid-encapsulated ODNs a punctate uptake pattern of TRITC-labeled ODNs was more apparent, which may indicate association of ODN–liposome complex with lysosomes (unpublished data). In addition to liposomal encapsulation, conjugation of ODNs with cholesterol or poly(L-lysine) has also been proposed as a potential method to improve cellular uptake and modify intracellular distribution of ODNs *in vitro* (Boutorine and Kostina, 1993; Clarenc *et al.*, 1993; Gryaznov and Lloyd, 1993). To date,

however, neither of these has been tested in *in vivo* neural systems.

Determination of specificity of behavioral effects of antisense DNA

To prove the specificity of antisense DNA effects, it is necessary to compare the behavioral effects of antisense ODNs with those of control ODNs; it is now required in most cases to use at least two control ODNs, e.g. sense, mismatch or scrambled sequences, in addition to antisense ODNs as well as showing changes in targeted gene products (Stein and Krieg, 1994). Use of a vehicle control group is also recommended in some studies (McCarthy *et al.*, 1993a, 1994b) since repeated or continuous treatment with ODNs, especially with S-ODNs, might have non-specific toxic effects. Relatively strong non-sequence-specific actions on protein synthesis are reported at higher concentrations of S-ODNs in *in vitro* systems (Crooke, 1991). It should be noted that for continuous ICV infusion *in vivo* (7 days, 1 μl/h) with an osmotic minipump, a concentration of up to 15 mM D-ODNs was tolerated without any neurologic or systemic toxicity, whereas only 1.5 mM was tolerated with S-ODNs (Whitesell *et al.*, 1993).

In addition to comparing the effects of ODNs composed of different nucleotide sequences, specificity of effects of antisense ODNs can be further determined by assessing their effects (i) on multiple behaviors (behavioral specificity); (ii) in different brain sites (anatomical specificity), (iii) at different time points after application (temporal specificity); and/or (iv) with agonists for different receptor subtypes (pharmacological specificity).

It was found that pretreatment with antisense ODNs for progesterone receptor mRNA in estrogen-primed ovariectomized females rats resulted in a great reduction in the progesterone-mediated lordosis quotient (decreased by 73% versus scrambled sequence ODNs), lordosis reflex intensity (44% decrease) and proceptive behaviors (80% decrease) whereas antisense ODNs had no effect on rejection (kicking and boxing) or vocalization (Ogawa *et al.*, 1994). In estrogen-primed ovariectomized females rats it was likewise found that the lordosis quotient was reduced by antisense ODNs for oxytocin receptor mRNA while antisense ODN-treated females showed more rejection (McCarthy *et al.*, 1994a). This study also showed that these behavioral effects of antisense ODNs were not simply due to damage at the injection site (hypothalamic ventromedial nucleus), since antisense ODN-treated females showed decreased levels of feeding behavior rather than the increased feeding behavior which would be expected in ventromedial nucleus-damaged rats.

Anatomical specificity of antisense ODNs was demonstrated by infusing antisense ODNs for glutamic acid decarboxylase mRNA into three different brain sites (McCarthy *et al.*, 1994b). Lordosis behaviors in estrogen-primed ovariectomized rats were reduced by antisense glutamic acid decarboxylase ODNs injected in the ventromedial nucleus of the hypothalamus or midbrain central gray, where GABA-A receptor agonist and antagonist are known to facilitate and inhibit lordosis respectively. In contrast, no inhibition of behavior was observed when the same antisense ODNs was administered into the preoptic area, in which GABA-A receptor agonist inhibited the behavior.

Onset, maintenance and extinction of behavioral effects of antisense ODNs are dependent on time after application. Most importantly, a number of studies have reported the recovery of behavioral effects after the termination of antisense ODN treatment (e.g. Chiasson *et al.*, 1992; McCarthy *et al.*, 1994b; Sakai *et al.*, 1994; Standifer *et al.*, 1994), suggesting that the blockade of gene expression and function are reversible. For example, Standifer *et al.* (1994) have shown in mice that inhibition of δ receptor agonist-mediated analgesia by antisense ODNs administered intrathecally three times every other day (days 1, 3 and 5) was greatest on day 6 (~80% compared with four bases-mismatchd ODNs or vehicle) but had recovered by 5 days after the last injection (day 10).

Finally, in many studies aimed to block agonist action by pretreatment with antisense ODNs for mRNA of specific receptors, specificity has been determined by comparing behavioral effects of agonists for different receptor subtypes as described above, e.g. D_1- and D_2-dopamine receptor agonists on locomotor and grooming behaviors (Weiss *et al.*, 1993; Zhang and Creese, 1993; Silvia *et al.*, 1994; Zhang *et al.*, 1994; Zhou *et al.*, 1994); μ, δ and κ opioid receptor agonists on analgesia (Chien *et al.*, 1994; Lai *et al.*, 1994; Standifer *et al.*, 1994), etc.

Comments on discrepancies of antisense ODN effects on behaviors and protein levels

As discussed above, it is necessary to show parallel reduction of targeted gene products (protein levels) to help prove the specificity of behavioral effects by antisense ODN treatment. We have frequently observed, however, that antisense ODN treatments result in a much larger reduction of behaviors than of protein levels as determined by receptor binding assays or immunocytochemistry [although comparable reduction of protein levels was reported in some studies (e.g. Mani *et al.*, 1994a; Mani *et al.*, 1994b; Zhang and Creese, 1993)]. For example, the reduction of numbers of progesterone receptor immunoreactive cells in the antisense ODN-treated side of the hypothalamic ventromedial nucleus was a maximum of 40% compared with the scrambled sequence ODN-treated side, while progesterone-mediated sexual behaviors in female rats were decreased by 73–80% by pretreatment with antisense ODNs (Ogawa *et al.*, 1994). D_2-dopamine receptor agonist-induced rotational behavior was reduced by 75% in antisense

ODN-treated mice compared with scrambled control sequence ODNs or saline-infused mice. These effects were accompanied by a 15–23% reduction of D_2 receptor levels in the dorsolateral part of the striatum (Weiss *et al.*, 1993; Zhou *et al.*, 1994). Infusion of antisense ODNs for neuropeptide Y mRNA to the hypothalamic arcuate nucleus reduced carbohydrate and fat intake by 65–70% compared with infusions of sense ODNs or vehicle, whereas protein levels of neuropeptide Y were reduced by 33–40% in this nucleus (Akabayashi *et al.*, 1994). Inhibition of δ receptor agonist-mediated analgesia by intrathecally administered antisense ODNs (~80%, compared with four bases-mismatch ODNs or vehicle) was accompanied by a 25–30% reduction of δ receptor agonist binding in the spinal cord (Standifer *et al.*, 1994). These findings may imply that the magnitude of occurrence of a specific behavior is not a simple linear function of protein levels. That is, reduction of protein levels beyond a certain threshold point by antisense ODN treatment may result in a sudden decrease in the behavior. It is also possible that a small nonspecific effect of antisense ODNs on general protein synthesis may add to the specific functional effects.

Conclusions

We have studied neural mechanisms of behaviors by examining the correlation between a specific behavior and various molecular processes in the CNS (Pfaff *et al.*, 1994). Recent progress in the direct manipulation of gene expression in the brain has enabled us to study causal relationships between molecular processes and behavior. For example, the neurotrophic viral vector method has been used successfully in this laboratory to manipulate gene expression in adult mammalian neural tissue (Kaplitt *et al.*, 1991, 1993). The antisense DNA method, as reviewed here, is also simple enough and has great potential for application to studies of molecular mechanisms underlying various kinds of behaviors. Furthermore, used in combination with knockout transgenic methods, the antisense DNA method may provide a broad new approach for the genetic analysis of brain and behavioral function.

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